

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

**HAPten-CARRIER CONJUGATES FOR USE IN DRUG-ABUSE
THERAPY AND METHODS FOR PREPARATION OF SAME**

5 REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of United States patent application 08/563,673 filed November 28, 1995, which is a continuation-in-part of United States 10 patent application Serial Number 08/414,971 filed March 30, 1995.

FIELD OF THE INVENTION

15 The present invention relates to treatment of drug abuse. More specifically, the present invention relates to methods of treating drug abuse using drug/hapten-carrier conjugates which elicit antibody responses and/or using the antibodies to the drug/hapten-carrier conjugates.

20

BACKGROUND OF THE INVENTION

The prevalence of drug use and abuse worldwide, especially in the United States, has reached epidemic 25 levels. There are a plethora of drugs, both legal and illegal, the abuse of which have become serious public policy issues affecting all strata of society with its obvious medical and social consequences. Some users live in an extremely high risk population associated with poverty 30 and illegal activity. Other users who might classify themselves as recreational users are at risk due to (a) properties of the drug(s) which make them addictive, (b) a predisposition of the user to become a heavy user or (c) a combination of factors including personal circumstances, 35 hardship, environment and accessibility. Adequate treatment

of drug abuse, including polydrug abuse, requires innovative and creative programs of intervention.

Two especially problematic drugs of addiction are cocaine and nicotine. Cocaine is an alkaloid derived from the leaves of the coca plant (*Erythroxylon coca*). In the United States alone, there currently are more than 5 million regular cocaine users of whom at least 600,000 are classified as severely addicted (Miller et al. (1989) *N.Y. State J. Med.* pp. 390-395; and Carroll et al. (1994) *Pharm. News.* 1:11-16). Within this population, a significant number of addicts actively are seeking therapy. For example, in 1990, 380,000 people sought medical treatment for cocaine addiction and the number is increasing.. At that time, it was estimated that 100,000 emergency room admissions per year involve cocaine use. The cumulative effects of cocaine-associated violent crime, loss in individual productivity, illness, and death is an international problem.

The lack of effective therapies for the treatment of cocaine addiction strongly suggests that novel approaches must be developed. Additional factors contributing to the lack of successful treatment programs is that patterns of cocaine abuse have varied with time. In an article entitled "1994 Chemical Approaches to the Treatment of Cocaine Abuse" (Carroll et al. (1994) *Pharm. News*, Vol. 1, No. 2), Carroll et al. report that since the mid-1980's, intravenous and nasal dosing of the hydrochloride salt (coke, snow, blow) and smoking of cocaine free-base (crack) have become common routes of administration, producing euphoria and psychomotor stimulation which last 30-60 minutes. Unlike some other abused drugs, cocaine can be taken in binges lasting for several hours. This behavior leads to addiction, and in some cases, to toxic consequences (Carroll et al., *Pharm. News, supra.*).

There are only very limited treatments for drugs of abuse and no effective long term treatments for cocaine addiction. Treatments include, but are not limited to, 5 counseling coupled with the administration of drugs that act as antagonists at the opioid receptors or drugs that try to reduce the craving associated with drug addiction. One approach to treatment is detoxification. Even temporary remissions with attendant physical, social and psychological 10 improvements are preferable to the continuation or progressive acceleration of abuse and its related adverse medical and interpersonal consequences (Wilson et al. in *Harrison's Principle of Internal Medicine* Vol. 2, 12th Ed., McGraw-Hill (1991) pp. 2157-8). More specifically, 15 pharmacological approaches to the treatment of cocaine abuse generally involve the use of anti-depressant drugs, such as desipramine or fluoxetine which may help manage the psychological aspects of withdrawal but, in general, do not directly affect the physiology of cocaine. (Kleber (1995) 20 *Clinical Neuropharmacology* 18:S96-S109). Further, their effectiveness varies widely (Brooke et al. (1992) *Drug Alcohol Depend.* 31:37-43). In some studies, desipramine reduced self-administration (Tella (1994) *College on Problems of Drug Dependence Meeting Abstracts*; Mello et al. 25 (1990) *J. Pharmacol. Exp. Ther.* 254:926-939; and Kleven et al. (1990) *Behavl. Pharmacol.* 1:365-373), but abstinence rate following treatment did not exceed 70% (Kosten (1993) *Problems of Drug Dependence, NIDA Res. Monogr.* 85). There has also been the use of drugs which potentiate dopaminergic 30 transmission, such as bromocriptine, but the benefits of such drugs are limited in part by toxicity (Taylor et al. (1990) *West. J. Med.* 152:573-577). New drugs aimed at replacing methadone for opioid addiction, such as buprenorphine, have also been used based on cross- 35 interference with the dopaminergic system, however only limited clinical study information is available (Fudula et

al. (1991) *NIDA Research Monograph*, 105:587-588). Buprenorphine has been reported to decrease cocaine self-administration (Carroll et al. (1991) *Psychopharmacology* 106:439-446; Mello et al. (1989) *Science* 245:859-862; and 5 Mello et al. (1990) *J. Pharmacol. Exp. Ther.* 254:926-939); however, cocaine abstinence rates following treatment generally do not exceed 50% (Gastfried et al. (1994) *College on Problems of Drug Dependence Meeting Abstracts*; and Schottenfeld et al. (1993) *Problems on Drug Dependence, NIDA* 10 *Res. Monogr.* 311).

Present therapies used to treat cocaine addicts have at least four major limitations leading to a very high rate of recidivism. First, and perhaps most fundamentally, the 15 contributing neurochemical events in cocaine abuse and addiction are complex (Carroll et al. (1994) *supra*.). As a result, single acting neuropharmacological approaches, such as inhibition of dopamine uptake, do not appear to be sufficient to overcome addiction. Second, the drugs 20 currently used in cocaine addiction treatments have significant side-effects themselves, limiting their utility. Third, drug therapy compliance is problematic among this patient population. Current therapies can require frequent visits to a health care provider and/or self-administration 25 of drugs designed to cure the addict of his habit. Because many of these drugs prevent the euphoria associated with cocaine, there is a strong disincentive to taking the drug. (Carroll, et al. (1994) *supra*.; Kosten et al. (1993) *Problems of Drug Dependence, NIDA Res. Monogr.* 132:85; 30 Schottenfeld et al. (1993) *Problems of Drug Dependence, NIDA Res. Monogr.* 132:311.) Fourth, because of the complex chemistries involved in pharmacological therapies, many of them may be incompatible with other therapies currently in use or in clinical trials. Finally, most of the 35 pharmacotherapy studies have been administered in context of low-intensity outpatient treatment programs and have not

been linked with intensive outpatient treatment or other psychosocial treatment that appears necessary for successful management of cocaine dependent patients. (Rao (1995) *Psychiatric Annals* 25(6):363-368).

5

Nicotine (1-Methyl-2-(3-pyridyl)pyrrolidine) is an alkaloid derived from the tobacco leaf. Nicotine use is widespread throughout the world and is legally available in many forms such as cigarettes, cigars, pipe tobacco, and smokeless (chewing) tobacco. Although the addictive nature of nicotine and the dangers of smoking have been known for many years (Slade et al. (1995) *JAMA* 274(3):225-233), cigarette smoking remains popular. An estimated 51 million Americans smoke and, according to the Center for Disease Control and Prevention, 420,000 people each year die from smoking related disorders.

The most popular nicotine delivery system is the cigarette. Cigarettes contain 6 to 11 mg of nicotine, of which the smoker typically absorbs 1 to 3 mg. The typical pack-per-day smoker absorbs 20 to 40 mg of nicotine each day, achieving plasma concentrations of 25 to 50 ng per milliliter. The plasma half life of nicotine is approximately two hours; the half life of the major metabolite cotinine is 19 hours. (Henningfield (1995) *The New England Journal of Medicine* 333(18):1196-1203).

Since nicotine is legally and widely available there is relatively low pressure against its use, unlike cocaine. Although a large percentage of addicted smokers have expressed a desire to stop smoking, and many actually try to stop, only 2 to 3 percent of smokers become nonsmokers each year. (Henningfield (1995) *supra.*). The high rate of recidivism in smokers who try to quit is indicative of the strong effect of nicotine dependence. (O'Brien et al. (1996) *Lancet* 347:237-240).

Nicotine addiction is a chronic, relapsing disorder. Nicotine targets the mesolimbic reward system eventually resulting in physiological dependence. Evidence suggests 5 that nicotine binds to the α -subunit of the nicotinic acetylcholine receptors in the central and peripheral nervous systems resulting in increased dopamine release. It is thought that increased numbers of nicotinic acetylcholine receptors in the brain enhance the physiological dependence 10 of nicotine (Balfour (1994) *Addiction* 89:1419-1423). These physiological effects of nicotine are powerful reinforcers of the psychological addiction. The nicotine users increased cognition and improved mood, as well as the negative effects associated with abstinence (i.e., 15 withdrawal symptoms), serve as powerful motivators for continued tobacco use.

The lack of effective therapies for nicotine dependence and the poor rate of success in those who try and quit its 20 use indicate that there is a strong need for a new therapy. Currently, the two most popular therapies are nicotine polacrilex ("nicotine gum") and transdermal-delivery systems ("nicotine patch"). These "replacement medications" act to deliver low amounts of nicotine to the user over a period of 25 time to slowly wean the nicotine user off the drug. It is thought that these methods reduce withdrawal symptoms and provide some effects for which the user previously relied on cigarettes (such as desirable mood and attentional states). (Henningfield (1995) *supra*.). These methods, however, 30 suffer from the drawbacks of low penetrance and recidivism of the non-motivated quitter. Moreover, negative effects have been reported by users of nicotine gum such as mouth irritation, sore jaw muscles, dyspepsia, nausea, hiccups and paresthesia. Reported adverse effects from the nicotine 35 patch include skin reactions (itching or erythema), sleep disturbance, gastrointestinal problems, somnolence,

nervousness, dizziness and sweating (Haxby (1995) *Am. J. Health-Syst. Pharm.* 52:265-281).

Experimental diagnostic approaches and therapies for treating drug addiction have been suggested in the literature which have yet to be practiced. For example, vaccination as a therapeutic approach for drug addiction has been described previously in principle. Bonese et al. investigated changes in heroin self-administration by a rhesus monkey after immunization against morphine (Bonese et al. (1974) *Nature* 252: 708-710). Bagasra et al. investigated using cocaine-KLH vaccination as a means to prevent addiction (*Immunopharmacol.* (1992) 23:173-179), although no conclusive results are produced and the methods used by Bagasra are in dispute. (Gallacher (1994) *Immunopharm.* 27:79-81). Obviously, if a conjugate is to be effective in a therapeutic regimen, it must be capable of raising antibodies that can recognize free cocaine or nicotine circulating *in vivo*. Cerny (WO 92/03163) describes a vaccine and immunoserum against drugs. The vaccine is comprised of a hapten bonded to a carrier protein to produce antibodies. Also disclosed is the production of antibodies against drugs, and the use of these antibodies in the detoxification of one who has taken the drug. Carrera et al., *Nature* 378:727-730 (1995) discloses the synthesis of a cocaine-KLH vaccine to induce anti-cocaine antibodies which block the locomotor effects of the drug in rats. Blincko, U.S. Patent No. 5,256,409, discloses a vaccine comprising a carrier protein bound to one hapten from the desipramine/imipramine class of drugs and another hapten from the nortriptyline/amitriptyline class of drugs. Liu et al., U.S. Patent No. 5,283,066, discloses use of a hapten-polymeric solid support complex to induce an immune response.

Passive administration of monoclonal antibodies to treat drug abuse has been previously described (see, Killian et al. (1978) *Pharmacol. Biochem. Behavior* 9:347-352; Pentel et al. (1991) *Drug Met. Dispositions* 19:24-28). In this approach, pre-formed antibodies to selected drugs are passively administered to animals. While these data provide a demonstration of the feasibility of immunological approaches to addiction therapy, passive immunization as a long term human therapeutic strategy suffers from a number of major drawbacks. First, if antibodies to be used for passive therapy are from non-human sources or are monoclonal antibodies, these preparations will be seen as foreign proteins by the patient, and there may be a rapid immune response to the foreign antibodies. This immune response may neutralize the passively administered antibody, blocking its effectiveness and drastically reducing the time of subsequent protection. In addition, readministration of the same antibody may become problematic, due to the potential induction of a hypersensitivity response. These problems can be overcome by production of immune immunoglobulin in human donors immunized with the vaccine. This approach is discussed in more detail in the Examples. Second, passively administered antibodies are cleared relatively rapidly from the circulation. The half life of a given antibody *in vivo* is between 2.5 and 23 days, depending on the isotype. Thus, when the antibodies are passively administered, rather than induced by immunization, only short term effectiveness can be achieved.

Another immunological approach to drug addiction has been to use a catalytic antibody which is capable of aiding hydrolysis of the cocaine molecule within the patient (Landry et al. (1993) *Science* 259:1899-1901). The catalytic antibody is generated by immunization of an experimental animal with a transition state analog of cocaine linked to a carrier protein; a monoclonal antibody is then selected that

has the desired catalytic activity. Although this approach is attractive theoretically, it also suffers from some serious problems. Catalytic antibodies must be administered passively and thus suffer from all of the drawbacks of 5 passive antibody therapy. Active immunization to generate a catalytic antibody is not feasible, because enzymatic activity is rare among antibodies raised against transition state analogs, and activity does not appear to be detectable in polyclonal preparations. In addition, the general 10 esterase-like activity of such catalytic antibodies and the uncontrolled nature of the active immune response in genetically diverse individuals makes them potentially toxic molecules, particularly when they are being produced within a human patient.

15

Yugawa et al. (EP 0 613 899 A2) suggest the use of cocaine-protein conjugate containing a cocaine derivative for raising antibodies for the detection of cocaine or cocaine derivatives in a blood sample. The Syva patents 20 (U.S. Patent No. 3,888,866, No. 4,123,431 and No. 4,197,237) describe conjugates to raise cocaine antibodies for immunoassays. Disclosed are conjugates to BSA using diazonium salts derived from benzoyl ecgonine and cocaine. Conjugates are made using para-imino ester derivatives of 25 cocaine and norcocaine to conjugate a carrier. Biosite (WO 93/12111) discloses conjugates of cocaine using the para-position of the phenyl ring of various cocaine derivatives increasing stability to hydrolysis by introducing an amide bond. The Strahilevitz patents (U.S. Patent No. 4,620,977; 30 U.S. Patent 4,813,924; U.S. Patent 4,834,973; and U.S. Patent 5,037,645) disclose using protein conjugates of endogenous substances and drugs for treatment of diseases, preventing dependence on psychoactive haptens, as well as 35 for use in immunoassays, immunodialysis and immunoadsorption.

Bjerke et al. (1987) *Journal of Immunological Methods* 96:239-246 describes the use of a conjugate of cotinine 4'-carboxylic acid bound covalently to poly-L-lysine to generate antibodies to the nicotine metabolite cotinine for use in determining the presence of cotinine in physiological fluids. Additionally, Abad et al. (1993) *Anal. Chem.* 65(22):3227-3231 describe the use of 3'-(hydroxymethyl) nicotine hemisuccinate conjugated to BSA to generate antibodies to nicotine for use in an ELISA used to measure nicotine in smoke condensates of cigarettes. Neither reference, however, teaches or suggests the use of a nicotine-carrier conjugate for use as a vaccine against nicotine abuse.

No effective therapy for drug addiction, especially, cocaine and nicotine addiction, has been developed. Thus, there is a need to develop a long term treatment approach to drug addiction, in particular cocaine and nicotine addiction, which does not depend totally on the addicted individual for compliance and self-administration.

SUMMARY OF THE INVENTION

The present invention overcomes the above mentioned drawbacks and provides methods for treating drug abuse. Using therapeutic compositions, in particular hapten-carrier conjugates, the present invention elicits an immune response in the form of anti-drug antibodies within the addict which upon subsequent exposure to the drug in a vaccinated individual neutralizes the drug so the expected pharmacological effects are diminished, if not eliminated. The present invention provides a therapeutic for drug addiction, particularly cocaine and nicotine addiction, based on vaccination of subjects with a drug/hapten-carrier conjugate, and more particularly, a cocaine-protein or nicotine-protein conjugate. Therapeutic compositions of the

invention comprise at least one hapten and at least one T cell epitope-containing carrier which when conjugated to form a hapten-carrier conjugate is capable of stimulating the production of anti-hapten antibodies. The hapten can be 5 a drug or drug derivative, particularly cocaine or nicotine. When the therapeutic composition containing the drug/hapten-carrier conjugate is administered to an addicted individual, anti-drug antibodies specific to the drug are elicited. A therapeutic immunization regimen elicits and maintains 10 sufficiently high titers of anti-drug antibodies, such that upon each subsequent exposure to the drug during the period of protection provided by the therapeutic, anti-drug antibodies neutralize a sufficient amount of the drug in order to diminish, if not eliminate, the pharmacological 15 effect of the drug. Also provided are novel methods of preparing these conjugates. A method of passive immunization is also provided, wherein a subject is treated with antibodies generated in a donor by vaccination with the hapten-carrier conjugate of the invention.

20

These and other features, aspects and advantages of the present invention will become more apparent and better understood with regard to the following drawings, description, and appended claims.

25

30

35

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a schematic representation of the structural formula of cocaine.

5

Figure 1b is a diagram representing sites of variability when preparing a cocaine conjugate of the instant invention. The sites of variability are arbitrarily assigned to easily designate the compound and conjugates of the instant invention and not necessarily reaction sites.

10
15
Figure 2a is a representation of a number of possible, arbitrarily labelled, "branches" of a hapten-carrier conjugate identified for ease of understanding suitable compounds and conjugates used in the practice of the instant invention.

20
Figure 2b is a representation of a number of possible, arbitrarily labelled, "branches" of a hapten-carrier conjugate identified for ease of understanding suitable compounds and conjugates used in the practice of the instant invention, wherein Q' is a modified T-cell epitope-containing carrier, such as a modified protein carrier.

25
Figure 3a is a representation of 6 cocaine conjugates (PS-2, PS-3, PS-4, PS-5, PS-6, and PS-9) of the instant invention, where Q is a T cell epitope-containing carrier such as a carrier protein or modified T cell epitope-containing carrier such as a modified carrier protein.

30

Figure 3b is a representation of "branches" at the sites of variability off the tropane ring of cocaine of the cocaine conjugates and intermediates of the instant invention. Figure 4 is a representation of "branches" at the sites of variability off the tropane ring in Figure 1b

of four compounds useful in preparing the conjugates of the instant invention.

Figure 5 is a representation of the structures of five reagents useful in the practice of the instant invention.

Figure 6 is a representation of the structures of four alternative drugs of abuse suitable for conjugation and administration in accordance with the teachings of the instant invention.

Figure 7 is a schematic diagram representing two possible conjugation reactions to prepare a single cocaine conjugate (PS-5) according to the methods of the instant invention.

Figure 8 is a representation of the structures of "succinylated norcocaine" and "pre-activated succinylated norcocaine" useful in the preparation of some of the conjugates of the instant invention.

Figure 9a is a graph showing the IgG antibody response in mice immunized with cocaine conjugate (PS-5.1/.6 + CFA i.p.) of the instant invention. The antibody response is detected by *in vitro* binding to the appropriate HEL conjugate made using HEL rather than BSA as a carrier. Mice received 2 injections of 50 µg per injection. The curves represent the response of 5 individual mice per group.

Figure 9b is a graph showing the IgG antibody response in mice immunized with cocaine conjugate (PS-5.5 Alum i.p.) of the instant invention. The antibody response is detected by *in vitro* binding to the appropriate HEL conjugate made using HEL rather than BSA as a carrier. Mice received 2 injections of 50 µg per injection. The curves represent the response of 5 individual mice per group.

Figure 9c is a graph showing the IgG antibody response in mice immunized with cocaine conjugate (PS-9.2 + CFA i.p.) of the instant invention. The antibody response is detected by *in vitro* binding to the appropriate HEL conjugate made using HEL rather than BSA as a carrier. Mice received 2 injections of 50 μ g per injection. The curves represent the response of 5 individual mice per group.

10 Figure 10a is a graph demonstrating that antiserum binding to a cocaine-protein conjugate can be competed off using free cocaine.

Figure 10b is a bar graph showing that immune antiserum can bind ³H-cocaine.

Figure 11a is a bar graph illustrating that a cocaine-BSA conjugate prepared according to the method of the instant invention provide two-fold protection in high dose cocaine LD₅₀.

Figure 11b is another bar graph illustrating that a cocaine-BSA conjugate prepared according to the method of the instant invention provide two-fold protection in high dose cocaine LD₅₀.

Figure 12a is a representation of a gel showing the relative molecular weights of native (monomer and pentamer) and recombinant cholera toxin-B (CTB) (monomer).

30 -

Figure 12b is a representation of a gel illustrating the stability of CTB pentamers over a pH range of 3 - 9.

Figure 12c is a drawing of a Western Blot gel showing peak fractions rCTB#32 and rCTB#53 which were obtained by periplasmic expression resulting in pentameric CTB.

Figure 13a is a graph representing an ELISA where the anti-CTB antibody detects the ability of rCTB to bind to ganglioside GM1 on the ELISA plate.

5

Figure 13b is a scan depicting a flow cytometry binding assay in which rCTB is bound to eukaryotic cells expressing ganglioside GM1.

10 Figure 14a is a graph representing an ELISA in which native CTB and cocaine-CTB conjugate CTB-5.8 (PS-5.8 conjugated to CTB) are shown to be pentameric, based on their ability to bind to ganglioside GM1.

15

Figure 14b is a graph representing an ELISA in which CTB-5.8 (PS-5.8 conjugated to CTB) is bound to ganglioside GM1 and the conjugate is detected with an anti-cocaine (anti-benzoylecgonine) monoclonal antibody.

20

Figure 15 is a schematic representation of another reaction useful in the preparation of conjugates of the instant invention, in particular, 3 benzoate ester adduct 4.

25

Figure 16 is a schematic representation of the synthesis of a carbon-13 labelled conjugate.

Figure 17a is a schematic representation of the structural formula of nicotine.

30

Figure 17b is a diagram representing sites of variability when preparing a nicotine conjugate of the instant invention. The sites of variability are arbitrarily assigned to easily designate the compound and conjugates of the instant invention and not necessarily reaction sites. These sites of variability are as referred to in Figure 18.

Figure 18 is a representation of "branches" at the sites of variability off the nicotine molecule for nicotine conjugates and intermediates of the instant invention.

5 Nicotine conjugates of the present invention are represented when Q is a T cell epitope containing carrier.

Figure 19 is a representation of nicotine metabolites useful in preparation of some of the conjugates of the 10 present invention.

Figure 20 shows succinyl norocaine consisting of a mixture of at least two isomers; namely the exo and endo forms of the succinyl group.

15 Figure 21 shows results of ¹H-NMR analysis demonstrating that the ratio of exo:endo for succinyl norcocaine is dependent upon the dielectric constant (ϵ) of the solvent used.

20 Figure 22 shows the proposed basis for the marked increase in stability of norcocaine over cocaine, which may be thought of as being due to the inability of the tropane nitrogen to stabilize the hydrolysis intermediate.

25 Figure 23 shows results of testing of different drugs at varying concentrations for their ability to inhibit the binding of antibodies to cocaine-HEL. The panel of drugs tested included cocaine, benzoylecgonine (the major 30 metabolite of cocaine); dopamine, serotonin, and norepinephrine (neurotransmitters); methylphenidate and amphetamine (CNS stimulators); procainamide HCl (a cardiac depressant); atropine (a compound that has a tropane ring in its structure); and lidocaine (a general anesthetic). The 35 pool of anti-cocaine antisera was specific for cocaine in

that cocaine competed with the cocaine-HEL conjugate for binding to the antibodies.

Figure 24 shows results of testing of mouse sera in an ELISA for antibody binding to a conjugate of PS-55 and hen egg lysozyme protein (HEL). The mice had been immunized with a nicotine-BSA conjugate.

Figure 25A-C shows that in sera from mice which were injected with PS-55 BSA, antibody binding to PS-55 HEL was inhibited by free nicotine.

Figure 26 shows results of testing in an ELISA using plates coated with PS-5.4 conjugated to HEL (hen egg lysozyme) sera from Wistar male rats which were immunized with 10ug of cocaine-rCTB conjugate precipitated on alum intramuscularly and again bled 14 days after the second injection.

Figure 27 shows results of a test to directly determine whether the antibodies generated in rats are capable of recognizing the free cocaine molecule, using a competition ELISA.

DETAILED DESCRIPTION OF THE INVENTION

The patent and scientific literature referred to herein
5 establishes the knowledge that is available to those skilled
in the art. The issued U.S. Patents, PCT publications, and
other publications cited herein are hereby incorporated by
reference.

10 The present invention provides a therapeutic for drug
addiction, based on vaccination of an addicted individual
with a drug/hapten-carrier conjugate, and more particularly,
a cocaine-protein conjugate or a nicotine-protein conjugate.
Therapeutic compositions of the invention comprise at least
15 one hapten and at least one T cell epitope containing
carrier which when conjugated to form a hapten-carrier
conjugate is capable of stimulating the production of anti-
hapten antibodies. As used herein the term "T cell epitope"
refers to the basic element or smallest unit of recognition
20 by a T cell receptor, where the epitope comprises amino
acids essential to receptor recognition. Amino acid
sequences which mimic those of the T cell epitopes and which
modify the allergic response to protein allergens are within
the scope of this invention. A "peptidomimetic" can be
25 defined as chemical structures derived from bioactive
peptides which imitate natural molecules. The hapten can be
a drug such as cocaine, nicotine or drug derivative.

When the therapeutic composition containing the hapten/
30 drug (or derivative thereof) is administered to the addicted
individual, anti-drug antibodies specific to the drug are
elicited. A therapeutic immunization regimen elicits and
maintains sufficiently high titers of anti-drug antibodies,
such that upon subsequent exposure to the drug, neutralizing
35 antibodies attach to a sufficient amount of the drug in
order to diminish, if not eliminate, the pharmacological

effects of the drug. For example, when the therapeutic composition is a cocaine-carrier conjugate, treatment induces an anti-cocaine antibody response which is capable of reducing or neutralizing cocaine in the bloodstream or 5 mucosal tissue of a subject, thereby blocking the psychologically addictive properties of the drug. Since in the present invention delayed or reduced levels of the drug of abuse reach the central nervous system, the addict receives diminished or no gratification from the use of 10 cocaine. This same mechanism of action, when administering a nicotine-carrier conjugate, will induce anti-nicotine antibodies and diminish or extinguish the gratification from the use of nicotine. No side effects are expected from the administration of the therapeutic of the instant invention. 15 For example, the instant drugs-of-abuse are small and monovalent and so are not able to cross-link antibody. Therefore, formation of immune complexes and the associated pathologies are not expected to occur after exposure to the drug of abuse. It is now, and is expected to be, compatible 20 with current and future pharmacological therapies. Further, effective neutralization is long lasting. For example, neutralizing antibody responses against pathogens are known to last for years. Accordingly, it is expected that high-titer anti-drug antibodies elicited using the therapeutic 25 composition of the instant invention can be maintained for long periods of time and possibly, at least a year. This long-term effect of the therapeutic composition with reduced compliance issues reduces recidivism which is a problem with current therapies.

30

Additionally, the therapeutic vaccination approach of the present invention to cocaine addiction is compatible with other therapies currently in use or in clinical trials. In fact, early phase co-therapy is highly desirable because 35 of the time necessary to achieve optimal antibody titers. A number of diverse pharmacological agents would be suitable

as co-therapies in preventing cocaine relapse, for example, desipramine, buprenorphine, naloxone, haloperidol, chlorproazine, bromocriptine, ibogaine, mazindol, as well as others that may become relevant.

5

Similarly, the therapeutic vaccination approach of the present invention to nicotine addiction is compatible with other therapies for minimizing symptoms of nicotine withdrawal. For example, the nicotine-carrier conjugate of 10 the present invention may be used in conjunction with clonidine, buspirone, and/or antidepressants or sedatives. The vaccine produced by this approach will be compatible with the current nicotine replacement therapies, i.e., gums and patches. Since anti-nicotine antibodies would take 15 several weeks to be generated, some level of craving control would be provided by the use of nicotine replacement therapies.

The following are terms used herein, the definitions of 20 which are provided for guidance. As used herein a "hapten" is a low-molecular-weight organic compound that reacts specifically with an antibody and which is incapable of inciting an immune response by itself but is immunogenic when complexed to a T cell epitope-containing carrier 25 forming a hapten-carrier conjugate. Further, the hapten is characterized as the specificity-determining portion of the hapten-carrier conjugate, that is, it is capable of reacting with an antibody specific to the hapten in its free state. In a non-immunized addicted subject, there is an absence of 30 formation of antibodies to the hapten. The therapeutic composition is used to vaccinate individuals who seek treatment for addiction to drugs. In the instant invention, the term hapten shall include the concept of a more specific drug/hapten which is a drug, an analog of a portion of the 35 drug, or drug derivative. The therapeutic composition, or therapeutic anti-drug vaccine, when initially administered

will give rise to a "desired measurable outcome". Initially, the desired measurable outcome is the production of a high titer of anti-drug antibodies (approximately 0.1 mg/ml to 1 mg/ml of specific antibody in the serum).
5 However, manipulation of the dosage regimen suitable for the individual gives and maintains a sustained desired therapeutic effect. The "desired therapeutic effect" is the neutralization of a sufficient fraction of free drug of abuse to reduce or eliminate the pharmacological effects of
10 the drug within a therapeutically acceptable time frame by anti-drug antibodies specific for the drug upon a subsequent exposure to the drug. Determining the therapeutically acceptable time frames for how long it takes to get a sufficient antibody response to a given drug and how long
15 that antibody response is maintained thereto are achieved by those skilled in the art by assessing the characteristics of the subject to be immunized, drug of abuse to be neutralized, as well as the mode of administration. Using this and other vaccination protocols as a model, one skilled in that art would expect the immunity or the period of
20 protection to last several months, up to more than one year.

"Passive immunization" is also disclosed which encompasses administration of or exposure to intact anti-drug antibody or polyclonal antibody or monoclonal antibody fragment (such as Fab, Fv, (Fab')₂ or Fab') prepared using the novel conjugates of the instant invention. As stated above, passive immunization of humans with an anti-cocaine or anti-nicotine antibody of the present invention as a
25 stand-alone treatment may be less useful than active immunization. Passive immunization would be particularly useful as an initial co-treatment and/or a supplementary complementary treatment (for example, during the period of time after initial administration of the vaccine but before
30 the body's own production of antibodies) or in acute situations to prevent death (for example, when a person

presents with a drug overdose). In some situations, passive therapy alone may be preferable, such as when the patient is immunocompromised or needs a rapid treatment.

5 The drug-conjugates of the present invention, as well as the compositions of the present invention, may also be used as a prophylactic. That is, the drug-conjugates or compositions may be administered to a mammal prior to any exposure to the drug to generate anti-drug antibodies. The 10 generated anti-drug antibodies would be present in the mammal to bind to any drug introduced subsequent to the administration of the conjugate or composition, and therefore minimize or prevent the chance of becoming 15 addicted to the drug.

15 The therapeutic composition of the instant invention, and more specifically, the therapeutic anti-drug vaccine, is a composition containing at least one drug/hapten-carrier conjugate capable of eliciting the production of a 20 sufficiently high titer of antibodies specific to the drug/ hapten such that upon subsequent challenge with the drug/ hapten said antibodies are capable of reducing the addictive properties of the drug. The expected immune response to a 25 hapten-carrier conjugate is the formation of both anti- hapten and anti-carrier antibodies. The therapeutic level is reached when a sufficient amount of the anti-drug specific antibodies are elicited and maintained to mount a neutralizing attack on drug introduced after vaccination. The therapeutic regimens of the instant invention allow for 30 sufficient time for production of antibodies after initial vaccination and any boosting. Further, the optimal anti- drug vaccine contains at least one drug/hapten carrier conjugate comprising an optimal combination of the drug as hapten and a carrier so that production of anti-drug 35 antibodies is capable of achieving an optimal therapeutic level, that is, remaining *in vivo* at a sufficiently high

titer to withstand a subsequent challenge within several months with the selected drug. More particularly, the antibody titers remain sufficiently high to provide an effective response upon subsequent exposure to the drug for about two months to about one year or more depending upon the individual, more usually at least three months. This optimal composition consists of a hapten-carrier conjugate, excipients and, optionally adjuvants.

10 When used in the treatment of cocaine, the present invention defines a hapten-carrier conjugate, wherein the hapten is cocaine or a cocaine derivative, which can be used to immunize mammals, particularly humans, to elicit anti-cocaine antibodies capable of binding free drug and preventing transit of the drug to the reward system in the brain thereby abrogating addictive drug-taking behavior. It is believed that cocaine affects the neuronal uptake of dopamine, norepinephrine, and serotonin. While not intending to exclude other modes of action, it is believed 20 that once cocaine enters the blood stream following inhalation (snorting or smoking) or intravenous administration, it rapidly crosses the blood-brain barrier where the intact cocaine binds to specific recognition sites located on the dopamine transporter of mesolimbocortical neurons, thereby inhibiting dopamine reuptake into presynaptic neurons. The euphoric rush is due to rapid build-up of dopamine in the synapse. The rapid action of cocaine presents problems unique to cocaine therapy. For this reason, cocaine remains the most complex and 30 challenging, and before the present invention, elusive drug for which therapy is sought. Although estimates vary, it is believed that following intranasal administration, changes in mood and feeling states are perceived within about 2 to 5 minutes, and peak effects occur at 10 to 20 minutes. Thus, 35 the active ingredient, the hapten-carrier conjugate, must be

capable of eliciting fast-acting antibodies. Cocaine free-base, including the free-base prepared with sodium bicarbonate (crack), has a relatively high potency and rapid onset of action, approximately 8 to 10 seconds following 5 smoking. An embodiment of the instant invention elicits antibodies capable of rapidly and specifically neutralizing cocaine within this time frame. Due to the route of the circulation, i.v. cocaine is intermediate in time of onset 10 of euphoria taking from about 30 seconds to about 1 minute. Thus, when used in the treatment of cocaine abuse, the 15 therapeutic hapten-carrier conjugate composition of the instant invention induce anti-cocaine antibodies which alter the physiological response to cocaine in humans. These antibodies possess the appropriate bioavailability and speed of binding that is required to neutralize cocaine *in vivo*. The Examples herein describe experiments done in mice to simulate alteration of response in mammals.

When used in the treatment of nicotine, the present 20 invention defines a hapten-carrier conjugate, wherein the hapten is nicotine or a nicotine derivative, which can be used to immunize mammals, particularly humans, to elicit anti-nicotine antibodies capable of binding free drug and preventing transit of the drug to the reward system in the 25 brain thereby abrogating addictive drug-taking behavior (e.g., smoking cigarettes). It is believed that nicotine binds to the α -subunit of the nicotinic acetylcholine receptors in the brain which results in an increase in dopamine release. It is thought that increased numbers of 30 nicotinic acetylcholine receptors in the brain enhance the physiological dependence of nicotine. As discussed above in relation to cocaine, anti-nicotine antibodies would presumably limit the distribution of nicotine across the blood-brain barrier to the brain, thus reducing its 35 pharmacological effects. Antibody intervention in the case of nicotine, however, may have some advantages over cocaine.

For example, there is some level of standardization with nicotine delivery; that is, each cigarette contains on average 9mg of nicotine of which 1-3mg are effectively dispensed during smoking. Additionally, the peak plasma concentration of nicotine is 25-50ng/ml which is significantly lower than that of cocaine (0.3-1 μ g/ml). This should provide an ideal opportunity for intervention with moderately high affinity antibodies.

10 Initial vaccination with the therapeutic haptен-carrier conjugate composition of the present invention creates high titers of haptен-specific antibodies *in vivo*. Periodic tests of the vaccinated subjects plasma are useful to determine individual effective doses. Titer levels are 15 increased and maintained through periodic boosting. It is anticipated that this therapeutic will be used in combination with current drug rehabilitation programs, including counseling. Further, the therapeutic compositions of the present invention may be aimed at a single drug or 20 several drugs simultaneously or in succession and may be used in combination with other therapies. For example, the therapeutic haptен-carrier conjugate compositions and methods of the instant invention are used without adverse interactions in combination with conventional 25 pharmacological approaches and previously discussed "short term" passive immunization to enhance the overall effect of therapy.

The therapeutic haptен-carrier conjugate composition of 30 the present invention is prepared by coupling one or more haptен molecules to a T cell epitope containing carrier to obtain a haptен-carrier conjugate capable of stimulating T cells (immunogenic) which leads to T cell proliferation and a characteristic release of mediators which activate 35 relevant B cells and stimulate specific antibody production. Antibodies of interest are those specific to the haptен

portion of the hapten-carrier conjugate (also called the hapten-carrier complex). Therapeutic compositions containing a combination of conjugates, either to the same drug (cross-immunization) or to multiple drugs (co-immunization) are disclosed. Such co-mixtures of conjugates of multiple drugs are particularly useful in the treatment of polydrug abuse.

In selecting drug suitable for conjugation according to 10 the instant invention, one skilled in the art would select drug with properties likely to elicit high antibody titers. However, if the chosen molecule is similar to those molecules which are endogenous to the individual, antibodies raised against such a molecule could cross-react with many 15 different molecules in the body giving an undesired effect. Thus, the drug to be selected as the hapten (drug/hapten) must be sufficiently foreign and of a sufficient size so as to avoid eliciting antibodies to molecules commonly found inside a human body. For these reasons, alcohol, for 20 example, would not be suitable for the therapeutic of the instant invention. The antibodies raised against the therapeutic composition are highly specific and of a sufficient quantity to neutralize the drug either in the blood stream or in the mucosa or both. Without limiting the 25 invention, the drugs which are suitable for therapeutic composition (not in order of importance) are:

Hallucinogens, for example mescaline and LSD;
Cannabinoids, for example THC;
30 Stimulants, for example amphetamines, cocaine,
phenmetrazine, methylphenidate;
Nicotine;
Depressants, for example, nonbarbiturates (e.g. bromides,
chloral hydrate etc.), methaqualone, barbiturates,
35 diazepam, flurazepam, phencyclidine, and fluoxetine;
Opium and its derivatives, for example, heroin, methadone,

morphine, meperidine, codeine, pentazocine, and propoxyphene; and "Designer drugs" such as "ecstasy".

5 Figure 6 shows the structure of four drugs suitable for conjugation according to the instant invention.

The carrier of the instant invention is a molecule containing at least one T cell epitope which is capable of 10 stimulating the T cells of the subject, which in turn help the B cells initiate and maintain sustained antibody production to portions of the entire conjugate, including the hapten portion. Thus, since a carrier is selected because it is immunogenic, a strong immune response to the 15 vaccine in a diverse patient population is expected. The carrier, like the hapten, must be sufficiently foreign to elicit a strong immune response to the vaccine. A conservative, but not essential, approach is to use a carrier to which most patients have not been exposed to 20 avoid the phenomenon of carrier-induced epitope suppression. However, even if carrier-induced epitope suppression does occur, it is manageable as it has been overcome by dose changes (DiJohn et al. (1989) *Lancet* 1415-1418) and other protocol changes (Etlinger et al. (1990) *Science* 249:423- 25 425), including the use of CTB (Stok et al. (1994) *Vaccine* 12:521-526). Vaccines which utilize carrier proteins to which patients are already immune are commercially available. Still further, carriers containing a large number of lysines are particularly suitable for conjugation 30 according to the methods of the instant invention. Suitable carrier molecules are numerous and include, but are not limited to:

Bacterial toxins or products, for example, cholera toxin B- 35 (CTB), diphtheria

toxin, tetanus toxoid, and pertussis toxin and filamentous hemagglutinin, shiga toxin, *pseudomonas* exotoxin;
5 Lectins, for example, ricin-B subunit, abrin and sweet pea lectin;
Sub virals, for example, retrovirus nucleoprotein (retro NP),
rabies ribonucleoprotein (rabies RNP), plant viruses (e.g. TMV, cow pea and "cauliflower mosaic viruses"),
10 vesicular stomatitis virus-nucleocapsid protein (VSV-N), poxvirus vectors and Semliki forest virus vectors;
Artificial vehicles, for example, multiantigenic peptides (MAP), microspheres;
Yeast virus-like particles (VLPs);
15 Malarial protein antigen;

and others such as proteins and peptides as well as any modifications, derivatives or analogs of the above.

20 To determine features of suitable carriers, initial experiments were performed using bovine serum albumin as a protein carrier. The protein has been ideal for animal experiments, as it is inexpensive and contains large numbers of lysines for conjugation. However, it is less appropriate
25 for human vaccination because the generation of anti-BSA antibodies has the potential to cause adverse responses. Thus, using the results of these experiments, the above-described criteria were applied to a large number of candidate carriers. The result is the list of carriers
30 described above suitable for the practice of the instant invention.

The carrier of a preferred embodiment is a protein or a branched peptide (e.g., multi-antigenic peptides (MAP)) or
35 single chain peptide. An ideal carrier is a protein or peptide which is not commonly used in vaccination in the

country in which the therapy is used, thereby avoiding the potential of "carrier induced epitopic suppression." For example, in the U.S., where standard childhood immunization includes diphtheria and tetanus, proteins such as tetanus toxoid and diphtheria toxoid, if unmodified, may be less desirable as appropriate carriers. Further, the carrier protein should not be a protein to which one is tolerant. In humans, this would exclude unmodified human serum albumin. Further, many food proteins would have to be carefully screened before use as a carrier. Again, in humans, bovine serum albumin would be less desirable as a carrier due to the beef in the diet of most humans. Still further, it is highly advantageous if the carrier has inherent immunogenicity/adjuvanticity. A delicate balance must be struck between the desire for immunogenicity of the carrier and the desire to maximize the anti-hapten antibody. Still further, the preferred carrier would be capable of both systemic response and response at the site of exposure. This is particularly true of cocaine and nicotine which are more frequently administered across mucosal membranes. The speed of response is especially critical where cocaine has been smoked. Accordingly, in the case of cocaine and nicotine, a preferred carrier elicits not only a systemic response but also a pre-existing mucosal antibody response. In such a mucosal response the reaction of antibodies with cocaine and/or nicotine would happen rapidly enough to counteract the drug before it begins circulating in the blood stream.

30 One such preferred carrier is cholera toxin B (CTB), a highly immunogenic protein subunit capable of stimulating strong systemic and mucosal antibody responses (Lycke (1992) *J. Immunol.* 150:4810-4821; Holmgren et al. (1994) *Am. J. Trop. Med. Hyg.* 50:42-54; Silburt et al. (1988) *J. Immun. Meth.* 109:103-112; Katz et al. (1993) *Infection Immun.* 61:1964-1971). This combined IgA and IgG anti-hapten

response is highly desirable in blocking cocaine that is administered nasally or by inhalation, and in blocking nicotine that is absorbed in the mouth and lungs. In addition, CTB has already been shown to be safe for human 5 use in clinical trials for cholera vaccines (Holmgren et al., *supra*; Jertborn et al. (1994) *Vaccine* 12:1078-1082; "The Jordan Report, Accelerated Development of Vaccines" 1993., *NIAID*, 1993).

10 Other useful carriers include those with the ability to enhance a mucosal response, more particularly, LTB family of bacterial toxins, retrovirus nucleoprotein (retro NP), rabies ribonucleoprotein (rabies RNP), vesicular stomatitis virus-nucleocapsid protein (VSV-N), and recombinant pox 15 virus subunits.

In yet another embodiment, various proteins derivatives, peptides fragments or analogs, of allergens are used are carriers. These carriers are chosen because they 20 elicit a T cell response capable of providing help for B cell initiation of anti-hapten antibodies. Examples of and methods of making allergen proteins and peptides and their sequences are disclosed in WO 95/27786 published October 19, 1995. An allergen which is particularly suitable as a 25 carrier is *Cryptomeria japonica*, more particularly, recombinant *Cry j 1*, the sequence of which has been published with slight variation. In countries other than Japan, *Cryptomeria japonica* is not prevalent. Therefore, *Cry j 1* allergen generally fits one of the criteria of a 30 suitable carrier, that is a carrier to which a subject has not been previously exposed.

Using the methods and compositions of the present invention, and more particularly, the techniques set out in 35 the Examples below, one skilled in the art links the

selected drug/hapten with the selected carrier to make the hapten-carrier conjugate of the instant invention.

In one embodiment of the present invention, the antibodies induced by the therapeutic composition act within the time it takes for the drug to travel from the lungs through the heart to the brain. The ability to elicit this antibody response requires the careful selection of the carrier molecule.

10

Production of Recombinant B Subunit of Cholera Toxin

Cholera toxin is the enterotoxin produced by *Vibrio cholerae* and consists of five identical B subunits with each subunit having a molecular weight of 11.6 KDa (103 amino acids) and one A subunit of 27.2 KDa (230 amino acids) (Finkelstein (1988) *Immunochem. Mol. Gen. Anal. Bac. Path.* 85-102). The binding subunit, CTB, binds to ganglioside GM1 on the cell surface (Sixma et al. (1991) *Nature* 351:371-375; Orlandi et al. (1993) *J. Biol. Chem.* 268:17038-17044). CTA is the enzymatic subunit which enters the cell and catalyzes ADP-ribosylation of a G protein, constitutively activating adenylate cyclase (Finkelstein (1988) *Immunochem. Mol. Gen. Anal. Bac. Path.* pp. 85-102). In the absence of the A subunit, cholera toxin is not toxic.

Others have disclosed the production of high level recombinant expression of CTB pentamers (L'hoir et al. (1990) *Gene* 89:47-52; Slos et al. (1994) *Protein Exp. Purif.* 5:518-526). While native CTB is commercially available, it is difficult to rule out contamination with CTA. Therefore, recombinant CTB has been expressed in *E. coli* and assays have been developed for its characterization. The cholera toxin construct was purchased from the American Type Culture Collection (pursuant to U.S. Patent 4,666,837). Recombinant CTB was cloned from the original vector.

(pRIT10810) into an expression plasmid (pET11d, Novagen) with an extra N-terminal sequence containing a His6 tag and expressed in *E. coli* to the level of 25 mg/liter of culture. The protein was purified over a Ni²⁺ column using standard techniques and analyzed on SDS-PAGE (see Figures 12a, b and c). The recombinant CTB is monomeric in this assay and is larger than the native CTB monomer due to the N-terminal extension.

10 Pentameric recombinant CTB was produced both with and without the His tag using the cDNA modified by PCR to include the *Pel b* leader sequence. A C-terminal Stop codon was inserted to remove the His tag. Both constructs were expressed in *E. coli* from the pET22b vector (Novagen). The 15 His tagged protein was purified by Ni²⁺ affinity chromatography as above (13 mg/L). The untagged recombinant CTB was purified by ganglioside GM1 column affinity chromatography as described (Tayot et al. (1981) *Eur. J. Biochem.* 113:249-258). Recombinant CTB pentamer was shown 20 to bind to ganglioside GM1 in an ELISA and reacted with pentamer-specific antibodies in Western blots and ELISA. Recombinant CTB is also available from other sources, such as SBL Vaccin AB.

25 The pentameric structure of CTB may be preferred for binding to ganglioside GM1. The pentamer is stable to SDS as long as the samples are not boiled, permitting pentamerization to be assessed by SDS-PAGE. The gel in Figure 12a demonstrates that the native CTB is a pentamer 30 and is readily distinguishable from the denatured monomeric CTB. Pentamer structure is maintained over a pH range from 4 to 9 (see Figure 12b), which facilitates a variety of conjugation chemistries. The recombinant CTB initially expressed is monomeric. One way to obtain pentameric CTB is 35 by making adjustments to express properly folded pentameric CTB. It has been found that cytoplasmic expression provides

a much higher level of monomeric CTB. One skilled in the art is aware of methods of folding monomeric CTB into pentameric CTB (see, e.g., L'hoir et al. (1990) *Gene* 89:47-52). An alternative to re-folding monomeric CTB to obtain pentameric CTB is periplasmic expression which resulted in pentameric recombinant CTB able to bind GM1-ganglioside by ELISA. Figure 13a and Figure 13b show the data supporting this finding. One skilled in the art may find several approaches for obtaining pentameric recombinant CTB have been described, including periplasmic expression with a leader (Slos et al., *supra*; Sandez et al. (1989) *Proc. Nat'l. Acad. Sci.* 86:481-485; Lebens et al. (1993) *BioTechnol.* 11:1574-1578) or post-translational refolding (L'hoir et al., *supra*; Jobling et al. (1991) *Mol. Microbiol.* 5:1755-1767).

Another useful carrier is cholera toxin which provides improved mucosal response over CTB. It has been reported that the enzymatically active A subunit adjuvant enhances activity (Liang et al. (1988) *J. Immunol.* 141:1495-1501; Wilson et al. (1993) *Vaccine* 11:113-118; Snider et al. (1994) *J. Immunol.* 153:647).

One aspect of achieving the conjugate of the instant invention involves modifying the hapten, sufficiently to render it capable of being conjugated or joined to a carrier while maintaining enough of the structure so that it is recognized as free state hapten (for example, as free cocaine or nicotine). It is essential that a vaccinated individual has antibodies which recognize free hapten (cocaine or nicotine). Radioimmunoassay and competition ELISA assay (Figures 10a and 10b) experiments, explained in more detail in the Examples, can measure antibody titers to free hapten. Antibodies of interest are hapten-specific antibodies and, in some embodiments, are cocaine-specific antibodies or nicotine-specific antibodies. It should be

recognized that principles and methods used to describe the preferred embodiments may be extended from this disclosure to a wide range of hapten-carrier conjugates useful in the treatment of a variety of drug addictions and toxic responses.

Conjugates

Preparation of the novel cocaine-carrier conjugates of the instant invention are derived from cocaine and cocaine metabolites, primarily derivatives of norcocaine, benzoyl ecgonine and ecgonine methyl ester. As used herein, the term "cocaine-carrier conjugate" encompasses a conjugate comprised of a carrier linked to a cocaine molecule, a modified cocaine molecule, or any metabolite of cocaine. Figure 4 shows a representation of the cocaine molecule as compared to these molecules. In the case of norcocaine and ecgonine methyl ester, the secondary amine and the secondary alcohol functional groups present in the two compounds respectively, are modified to provide a chemical linkage which enables attachment to a protein carrier. In the case of benzoyl ecgonine, the free acid is either used directly to attach to a carrier protein or is modified with a linkage to facilitate the same. Preparation of the novel nicotine-carrier conjugates of the present invention are derived from nicotine and nicotine metabolites. Figure 19 shows a representation of nicotine and some of its metabolites.

The length and nature of the hapten-carrier linkage is such that the hapten is displaced a sufficient distance from the carrier domain to allow its optimal recognition by the antibodies initially raised against it. The length of the linker is optimized by varying the number of $-\text{CH}_2-$ groups which are strategically placed within a "branch" selected from the group consisting of:

	CJ 0	Q
5	CJ 1	$(\text{CH}_2)_n \text{Q}$
	CJ 1.1	$\text{CO}_2 \text{Q}$
	CJ 1.2	COQ
10	CJ 2	$\text{OCO}(\text{CH}_2)_n \text{Q}$
	CJ 2.1	OCOCH=Q
15	CJ 2.2	OCOCH(O)CH_2
	CJ 2.3	$\text{OCO}(\text{CH}_2)_n \text{CH(O)CH}_2$
	CJ 3	$\text{CO}(\text{CH}_2)_n \text{COQ}$
20	CJ 3.1	$\text{CO}(\text{CH}_2)_n \text{CNO}$
	CJ 4	$\text{OCO}(\text{CH}_2)_n \text{COQ}$
25	CJ 4.1	$\text{OCO}(\text{CH}_2)_n \text{CNO}$
	CJ 5	$\text{CH}_2 \text{OCO}(\text{CH}_2)_n \text{COQ}$
	CJ 5.1	$\text{CH}_2 \text{OCO}(\text{CH}_2)_n \text{CNO}$
30	CJ 6	$\text{CONH}(\text{CH}_2)_n \text{Q}$
	CJ 7	$\text{Y}(\text{CH}_2)_n \text{Q}$
35	CJ 7.1	$\text{CH}_2 \text{Y}(\text{CH}_2)_n \text{Q}$

CJ 8	$\text{OCOCH}(\text{OH})\text{CH}_2\text{Q}$
CJ 8.1	$\text{OCO}(\text{CH}_2)_n\text{CH}(\text{OH})\text{CH}_2\text{Q}$
5 CJ 9	OCOC_6H_5
CJ 10	shown on Figure 2b
CJ 11	$\text{YCO}(\text{CH}_2)_n\text{COQ}$

10

and shown in Figures 2a and 2b herein. With regard to the above branches, n is an integer preferably selected from about 3 to about 20, more particularly about 3 to about 6; Y is preferably selected from the group consisting of S, O, and NH; and Q is preferably selected from the group consisting of:

- (1) -H
- 20 (2) -OH
- (3) -CH₂
- (4) -CH₃
- (4a) -OCH₃
- (5) -COOH
- 25 (6) halogen
- (7) protein or peptide carrier
- (8) modified protein or peptide carrier
- (9) activated esters, such as 2-nitro-4-sulfophenyl ester and N-oxysuccinimidyl ester
- 30 (10) groups reactive towards carriers or modified carriers such as mixed anhydrides, acyl halides, acyl azides, alkyl halides, N-maleimides, imino esters, isocyanate, isothiocyanate; or
- (11) another "branch" identified by its "CJ" reference number.

A T cell epitope containing carrier (e.g., a protein or peptide carrier) may be modified by methods known to those skilled in the art to facilitate conjugation to the hapten (e.g., by thiolation). For example with 2-iminothiolane (Traut's reagent) or by succinylation, etc. For simplicity, $(\text{CH}_2)_n\text{Q}$, where Q = H, 5 or by succinylation, etc. For simplicity, $(\text{CH}_2)_n\text{Q}$, where Q = H, may be referred to as $(\text{CH}_3)_n$, methyl or Me, however, it is understood that it fits into the motif as identified in the "branches" as shown in Figures 2a and 2b.

10 Further abbreviations of commercially obtainable compounds used herein include:

BSA = Bovine serum albumin
 DCC = Dicyclohexylcarbodiimide
 15 DMF = N,N -Dimethylformamide
 EDC (or EDAC) = N-Ethyl-N'-(3-(dimethylamino) propyl) carbodiimide hydrochloride
 EDTA = Ethylenediamine tetraacetic acid, disodium salt
 HATU = O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium
 20 hexafluorophosphate
 NMM = N-Methylmorpholine
 HBTU = 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
 TNTU = 2-(5-Norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
 25 PyBroP® = Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
 HOEt = N-Hydroxybenzotriazole

Further the IUPAC nomenclature for several named compounds 30 are:

Norcocaine:

3 β -(Benzoyloxy)-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester

Benzoyl ecgonine:

3 β -(Benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid

5 Cocaine:

3 β -(Benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester

Ecgonine methyl ester:

10 3 β -(Hydroxy)-8-methyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester

Nicotine

1-Methyl-2-(3-pyridyl)pyrrolidine

15

Cotinine

N-Methyl-2-(3-pyridyl)-5-pyrrolidone

20 Reactions

In one embodiment, precursors of the conjugates of the instant invention are synthesized by acylating ecgonine methyl ester with bromoacetyl bromide in DMF in the presence of two equivalents of diisopropylethylamine. The product is then coupled to the thiol group of a thiolated carrier protein to obtain a conjugate with the general structure of PS-2 (see Figure 3a and Example 1).

30 In another embodiment, precursors of the conjugates of the instant invention are synthesized by succinylating ecgonine methyl ester with succinic anhydride in DMF in the presence of one equivalent of triethylamine. The product is then coupled to the amino group of a lysine residue of a carrier protein to obtain a conjugate with the general structure of PS-4 (see Figure 3a and Example 2).

In yet another embodiment, precursors of the conjugates of the instant invention are synthesized by reacting norcocaine with succinic anhydride in methylene chloride in the presence of two equivalents of triethylamine. Alternatively, precursors of the conjugates of the instant invention are synthesized by reacting a solution of norcocaine monoactivated succinic acid and triethylamine to form succinylated norcocaine. In either case, the resulting succinyl norcocaine consists of a mixture of at least two isomers, namely the exo and endo forms of the succinyl group (see Figure 20). We have demonstrated using ¹H-NMR analysis that the ratio of exo:endo is dependent upon the dielectric constant (ϵ) of the solvent used (see Figure 21). The lower the dielectric constant, the higher the ratio of one isomer. The mixture is the result of a dynamic equilibrium process and the isomers are not readily separable.

Succinyl norcocaine can then be coupled to the ϵ -amino group of a lysine residue of a carrier protein using EDC to obtain a conjugate with the general structure of PS-5 (see Figure 3a and Method A of Example 3). Conjugates with the general structure of PS-5 may be obtained in an alternative set of reactions. In this alternative, the protein conjugation can be carried out using a pre-activated succinylated norcocaine derivative. That is, the intermediate can be isolated and characterized. The pre-activated succinylated norcocaine derivative is synthesized by reacting 4-hydroxy-3-nitrobenzene sulfonic acid sodium salt with succinylated norcocaine in the presence of dicyclohexylcarbodiimide (DCC) and DMF. The product is conjugated to the amino group of a lysine residue of a carrier protein to obtain a conjugate with the general structure of PS-5 (See Figure 3a and Example 7, Method B).

In still another embodiment, compounds of the instant invention are synthesized by reacting succinylated norcocaine with N-hydroxysuccinimide in the presence of ethyl chloroformate, N-methylmorpholine (NMM) and DMF. The product is then coupled to

the amino group of a lysine residue of a carrier protein to obtain a conjugate with the general structure of PS-5 (see Figure 3a and Example 7, Method C).

5 In another embodiment, compounds of the instant invention are synthesized by reacting thionyl chloride with succinylated norcocaine. The product is then conjugated to a carrier protein to obtain a conjugate with the general structure of PS-5 (see Figure 3a and Example 7, Method A).

10 In another embodiment, compounds of the instant invention are synthesized by reacting succinylated norcocaine with HATU in DMF and diisopropylethylamine (Carpino (1993) *J. Am. Chem. Soc.* 115:4397-4398). The product was added to an aqueous solution 15 containing the carrier protein to obtain a conjugate with the general structure PS-5 (see Figure 3a and Method A of Example 7).

20 In another embodiment, compounds of the instant invention are synthesized by reacting succinylated norcocaine with HBTU in DMF and diisopropylethylamine. The product was added to an aqueous solution containing the carrier protein to obtain a conjugate with the general structure PS-5 (see Figure 3a and Method B of Example 7).

25 In yet another embodiment, compounds of the instant invention are synthesized by reacting succinylated norcocaine with TNTU in DMF and diisopropylethylamine. The product was added to an aqueous solution containing the carrier protein to obtain a conjugate with the general structure PS-5 (see Figure 3a and 30 Method C and D of Example 7).

35 In still another embodiment, compounds of the instant invention are synthesized by reacting succinylated norcocaine with PyBroP in DMF and diisopropylethylamine. The product was added to an aqueous solution containing the carrier protein to obtain a

conjugate with the general structure PS-5 (see Figure 3a and Method E and F of Example 7).

Alternatively, compounds of the instant invention are synthesized by succinylating the carrier protein with succinic anhydride in borate buffer. The product is then coupled to norcocaine in the presence of EDC to obtain a conjugate with the general structure of PS-5 (see Figure 3a and Method B of Example 3).

In another embodiment, compounds of the instant invention are synthesized by reducing the free acid in benzoyl ecgonine to its corresponding primary alcohol, using borane-dimethylsulfide complex. The alcohol is reacted with succinic anhydride in DMF, the product of which is then conjugated to the free amino acid group of a carrier protein in the presence of EDC to obtain a conjugate with the general structure of PS-6 (see Figure 3a and Example 4).

In another embodiment, compounds of the instant invention are synthesized by conjugating benzoyl ecgonine to the amino group of a lysine residue of a carrier protein in the presence of EDC to obtain a conjugate with the general structure of PS-9 (see Figure 3a and Example 5).

The PS-5 analogs of CTB are synthesized using the protocols described in Example 6. The various methods described in Example 6 for synthesizing PS-5 analogs of CTB yield PS-5 analogs with different degrees of hapteneation. The degree of hapteneation can be determined by UV absorption or time of flight (TOF) mass spectral analysis. Table 2 shows that hapteneation was achieved using several conjugates (some with CTB as a carrier) made pursuant to the methods of the instant invention. Different batches are indicated by adding a decimal and a number thereafter, e.g., PS-5 batch 6 is PS-5.6. The haptene-carrier conjugates of the invention can be hapteneated to different degrees by using the

methods described in Example 6 as well as various methods of conjugation known to those skilled in the art, e.g., different choices of activating agents, different buffers, different reaction times, etc.. The amount of haptenation of the conjugate is limited, however, by the number of nucleophilic groups contained within the carrier.

In one embodiment, the precursor of the conjugates PS-54 were synthesized by acylating racemic nornicotine with succinic anhydride in methylene chloride in the presence of two equivalents of diisopropylethylamine. The product of this reaction is then coupled to the lysine residue of a carrier protein using HATU to obtain the conjugates PS-54 (see Example 26, method B).

In another embodiment, the precursors of PS-55, PS-56, PS-57 and PS-58 were synthesized by selectively alkylating the pyridine nitrogen in (S)-(-)-nicotine in anhydrous methanol, with ethyl 3-bromobutyrate, 5-bromoaleric acid, 6-bromohexanoic acid or 8-bromoocanoic acid respectively (see Example 27, methods A, B, C, and D). The products of these reactions were conjugated to a carrier protein using HATU to obtain the conjugates PS-55, PS-56, PS-57 and PS-58 (see Example 28, Method A).

25

TABLE 2

Conjugate	Carrier Protein	Haptens/ Monomer	Conjugation Method
30 PS-2.2	BSA	16	Ex 1
35 PS-4.3	BSA	24	Ex 4

	PS-5.1	BSA	4-20	Ex 3, Method A
	PS-5.4	BSA	29	Ex 3, Method A
5	PS-5.6	BSA	20	Ex 3, Method A
	PS-5.7	BSA	27	Ex 3, Method B
	PS-6.1	BSA	9	Ex 4
10	PS-9	BSA	1-2	Ex 5
	PS-9.2	BSA	7	Ex 5
	PS-5.6	CTB	1.25	Ex 6, Method A
15	PS-5.7	CTB	<1	Ex 7, Method A
	PS-5.8	CTB	1.9	Ex 6, Method A
20	PS-5.9	CTB	0.9-6.5	Ex 7, Method B
	PS-5.10	CTB	0.5-2.5	Ex 7, Method C
25	PS-11	CTB	1.0-7.8	Ex 6, Method A
	PS-5.53	CTB	3.4	Ex 6, Method A
	PS-5.70	CTB	NA	Ex 6, Method B
30	PS-5.168	rCTB	5.7-11.8	Ex. 6, Method L
	PS-5.174	rCTB	6.9-10	Ex. 6, Method L
35	PS-5.179	rCTB	5.8-11.8	Ex. 6, Method L

	PS-5.169	rCTB	5.3-7.7	Ex. 6, Method G
	PS-5.175	rCTB	4.0-7.7	Ex. 6, Method G
5	PS-5.180	rCTB	6.6-10.7	Ex. 6, Method G
	PS-5.185	rCTB	7.3-12	Ex. 6, Method G
	PS-5.170	rCTB	2.7-7.8	Ex. 6, Method M
10	PS-5.176	rCTB	1.0-5.6	Ex. 6, Method M
	PS-5.181	rCTB	1.7-5.4	Ex. 6, Method M
15	PS-5.184	rCTB	1	Ex. 6, Method M
	PS-5.171	rCTB	1.0-6.9	Ex. 6, Method H
	PS-5.177	rCTB	0.9-4.5	Ex. 6, Method H
20	PS-5.182	rCTB	3.2-607	Ex. 6, Method H
	PS-5.186	rCTB	3.8-6.5	Ex. 6, Method H
25	PS-5.187	rCTB	1.6-6.5	Ex. 6, Method I
	PS-5.189	rCTB	1	Ex. 6, Method I
	PS-5.194	rCTB	0.3-2.8	Ex. 6, Method I
30	PS-5.195	rCTB	0.9-3.0	Ex. 6, Method I
	PS-5.196	rCTB	0.6	Ex. 6, Method I
35	PS-5.200	rCTB	1.0-5.5	Ex. 6, Method I

	PS-54	BSA	19.5	Example 26, Method B
5	PS-54	HEL	3.2	Example 26, Method B
	PS-55	BSA	33.2	Example 28, Method A
10	PS-55	HEL	1.09	Example 28, Method A
	PS-56	BSA	27	Example 28, Method A
15	PS-56	HEL	2.2	Example 28, Method A
	PS-57	BSA	81	Example 28, Method A
20	PS-57	HEL	8	Example 28, Method A
	PS-58	BSA	66.8	Example 28, Method A
25	PS-58	HEL	7.4	Example 28, Method A

30

NA - not available

This is a non-limiting list of conjugates. Other conjugates have been made with greater than one hapten coupled to the T cell epitope-containing carrier. Preferably, 1 to 100 haptens are coupled to the T cell epitope-containing carrier. Most preferably, 1 to 70 haptens are coupled to the T cell epitope containing carrier.

Methods of synthesizing compounds PS-2, PS-3, PS-4, PS-5, PS-6, PS-9, PS-54, PS-55, PS-56, PS-57 and PS-58 are disclosed in 10 the Examples. Following the methods disclosed, e.g., using activating agents under aqueous conditions, one skilled in the art can synthesize compounds PS-10 to PS-53 (see Figure 3b(1) and (2)).

15 Hydrolysis of the methyl ester in the PS-2, PS-4, and PS-5 conjugates leads to the production of benzoyl ecgonine-specific antibodies, thus rendering the conjugate essentially ineffective as a therapeutic vaccine. For optimal conjugation and to prevent extensive hydrolysis of the methyl ester in the 20 succinylated norcocaine and PS-5 conjugates, the buffer pH during conjugation is carefully controlled. Landry, American Chemical Society, Division of Medicinal Chemistry, 212th ACS National Meeting, Abstract No. 161 (1996), indicates that the stability of the methyl ester in cocaine is lower than the 25 corresponding ester in pseudococaine and in N-acylated norcocaine, when studied under the same conditions. Since succinylated norcocaine is an N-acylated derivative of norcocaine, the stability of the methyl ester was investigated under basic conditions. HPLC analysis of succinylated 30 norcocaine at pH 6, 7 and 8 at 5°C indicates that hydrolysis is essentially undetectable over 24 hours. The time for 5% degradation at 25°C at the same pH values decreases from 1.30 years at pH 6 to 10.96 days at pH 8. Thus, the degradation follows first order kinetics and shows a strong pH dependency. 35 At higher pH ranges (e.g., pH 9 and 10) the rate of hydrolysis is faster.

This marked increase in stability over cocaine may be thought of as being due to the inability of the tropane nitrogen to stabilize the hydrolysis intermediate (see Figure 22). Our preferred conditions for formulating the conjugate are based upon its solubility and stability, along with the need to produce a sterile finished product. This preferred formulation is amenable to sterilization by filtration. The effect of pH on the solubility of the conjugate at 25°C was tested. The conjugate was quantitated using total nitrogen analysis. The equilibrium solubility of the conjugate is near 200 µg/ml at all pH values below 8.0. However, below pH 8, a very significant amount of the total conjugate (approximately 80%) is lost as a result of filter sterilization. To completely solubilize the conjugate for passage through a sterile filter, a pH value of at least 10 is preferred. Accordingly, preferred conditions for the filter sterilization process are filtration at pH 10.0 and 5°C for one hour or less. Preferably, such filtration is followed by adsorption of the conjugate onto sterile Alum to drop the formulation pH to physiologic values. The sterile conjugate-Alum suspension preferably is then filled into vials and stored at 5°C. Other buffers may be utilized to promote solubility below pH 10.

Methods to monitor the stability of the methyl ester can be both immunological and physiochemical. A cocaine-specific monoclonal antibody has been generated which can discriminate between cocaine and its metabolites when attached to the protein carrier. The reactivity to inactive metabolites was 2000 times less than to cocaine. Benzoylecgonine-specific monoclonal antibodies can be generated in-house using similar technology. Either monoclonal antibody or preferably both can be used to measure levels of intact and hydrolyzed conjugates compared to standard mixtures. This differentiation depends on the relative reactivity of each monoclonal antibody to the hydrolyzed and intact conjugate. In another embodiment a carbon-13 enriched

containing methyl ester analog of succinylated norcocaine can be synthesized (Figure 16). When conjugated to a carrier protein to form PS-5, carbon-13 nuclear magnetic resonance spectroscopy (¹³C NMR) can be used to monitor the presence of the methyl ester and since the methyl group is isotope enriched, the signal corresponding to the methyl ester will be distinguishable above the protein signals.

In another embodiment a radioactively labelled methyl ester containing conjugate can be synthesized. This could include either a carbon-14 or tritium containing methyl ester analog of succinylated norcocaine. When conjugated to a carrier protein to form PS-5, the loss of radioactivity from either analog over time can be monitored using techniques known to those familiar with the art. Monitoring the loss of radioactivity will then indicate the residual levels of intact methyl ester.

The benzoate ester group in the PS-5 conjugates is essentially stable under the conditions of conjugation and purification, and therefore requires no monitoring for retention of structural integrity. If, however, increased bioavailability is desirable then incorporation of an amide bond or some other metabolically stable group, known to those familiar with the art, can be incorporated into the conjugate. Similarly, the methyl ester in the PS-5 conjugates can be stabilized using the branch CJ6 where Q = H, i.e. an amide bond. This incorporation would increase both the *in vitro* and *in vivo* stability of the conjugates.

30

HPLC Analysis of CTB Cocaine Conjugates

Reverse phase HPLC is used as an in-process control to monitor the conjugation of succinylated norcocaine to recombinant cholera toxin B subunit (rCTB). This method shows how levels of hapteneation change with respect to reaction time

and differential levels of activating agent. In addition, byproducts of the reaction and residual small organic compounds, e.g. unreacted succinylated norcocaine, can be measured. Also the amount of unreacted rCTB may be quantitated with respect to the product. A reverse phase column was chosen in order that components of the product may be separated with respect to number of haptens per rCTB monomer as the product becomes increasingly hydrophobic with the addition of each succinylated norcocaine molecule.

Conjugate CTB-5.200 was analyzed by RP HPLC (as described in Example 30, Method A) to measure the retention time of the conjugate, the percentage of unconjugated rCTB, and the amount of residual unreacted succinylated norcocaine. This assay was performed five times on CTB-5.200 to demonstrate reproducibility of the instrument and the process. Using this process unreacted rCTB eluted at 24.1 minutes with an unresolved shoulder at 25.6 minutes. This could be accounted for by the heterogeneity of the rCTB amino terminus. The amount of unreacted rCTB in the conjugate was consistently less than 1% as measured at 210 nm. Conjugated material eluted as a broad multiplet of peaks beginning at 25.6 minutes and continuing to 37 minutes (data not shown). The amount of residual succinylated norcocaine was negligible (<0.1%).

The conjugate CTB5-200 was separated into various fractions using a semi-preparative method (see Example 30, Method B). Lyophilized fractions were resuspended in 20% acetonitrile 0.1% TFA and analyzed by mass spectrometer and analytical RP HPLC (as previously described in Example 30, Method A). The mass spectral analysis indicated that the conjugate had been fractionated by level of haptenation with later peaks showing higher levels of haptenation. Analytical RP HPLC revealed several peaks within each fraction, this was expected as baseline resolution was not achieved at the semi-preparative scale. There appeared to be a trend whereby the more highly

haptenated species had a longer retention time as compared to unconjugated material or even material containing only one hapten (data not shown). The first fraction collected at 24.1 minutes was found to be completely unreacted material whereas fraction four at 28.8 minutes was had 3.5 haptens/monomer. Moreover, fraction number eight, with an elution time of 39 minutes contained the most highly haptenated molecules (7.6 haptens/monomer). Chromatographic separation of the constitutive components of the conjugate appeared to be based on level of haptenation; although there remains the possibility that differentially haptenated residues may lead to variation in hydrophobicity and thus level of haptenation would not be the only basis for separation.

In yet another embodiment, compounds PS-27 to PS-50 are synthesized via a series of reaction which allow a novel entry into the tropane class of alkaloids. This novel route involves a free radical mediated 1,6 diene-like intermolecular cyclization (March, *Advanced Organic Chemistry: Reactions, Mechanisms and Structure*, (1992) 4th ed., Wiley-Interscience, p. 744, and references cited therein). Tropane alkaloids, in particular cocaine and its analogs, have been previously synthesized; however these routes involve multiple steps and usually resolution of an intermediate (Wilstatter et al. (1923) *Ann. Chem.* 434:111-139; Tufariello et al. (1979) *J. Am. Chem.* 101:2435-2442; Lewin et al. (1987) *J. Heterocyclic Chem.* 24:19-21; and Simoni et al. (1993) *J. Med. Chem.* 36:3975-3977). Although limited to the synthesis of 3-aryltropane derivatives, Davies et al. (U.S. Patent 5,262,428), synthesized cocaine analogs by decomposing vinyl diazoethanes in the presence of pyrroles to form a tropane ring which is then followed by a Grignard addition to provide the cocaine analogs. In this alternative embodiment, novel cocaine-carrier conjugates with "remote site" branches are synthesized. As used herein "remote sites" are labelled C, D and E on Figure 1. Those sites pose special challenges to the chemist due to the nature of the

tropane ring and are especially difficult positions for "branches" necessary for conjugates of the instant invention. One embodiment, adds the "branches" then builds the tropane ring last. As represented in Figure 15, there is a novel single step 5 addition of the radical 2 and cyclization of, at low temperature, general compound 1. The stereochemical outcome is defined by the boat-like form of the intermediate 3 in which addition of the radical 2 occurs equatorially at position 3 followed by ring closure by the predicted mechanism, which gives 10 the 3 -benzoate ester adduct 4 (cocaine analog). The orientation of C, D, E and CO₂R would be predefined in 1.

There is a wide range of compounds which have been developed to facilitate cross-linking of proteins/peptides or conjugation 15 of proteins to derivatized molecules, e.g., haptens. These include, but are not limited, to carboxylic acid derived active esters (activated compounds), mixed anhydrides, acyl halides, acyl azides, alkyl halides, N-maleimides, imino esters, isocyanates and isothiocyanates, which are known to those 20 skilled in the art. These are capable of forming a covalent bond with a reactive group of a protein molecule. Depending upon the activating group, the reactive group is the amino group of a lysine residue on a protein molecule or a thiol group in a carrier protein or a modified carrier protein molecule 25 which, when reacted, result in amide, amine, thioether, amidine urea or thiourea bond formation. One skilled in the art may identify further suitable activating groups, for example, in general reference texts such as *Chemistry of Protein Conjugation and Cross-Linking* (Wong (1991) CRC Press, Inc., Boca Raton, FL). 30 Ideally, conjugation is via a lysine side chain amino group. Most reagents react preferentially with lysine. An especially suitable carrier is CTB as it has 9 lysine residues per monomer in its native form. To determine if conjugated pentameric CTB retains its structure and activity, GM1 ganglioside binding can 35 be assessed.

Applicants have expressed and purified amounts of recombinant CTB which, once optimized, are produced in large fermentation batches. Processes for expressing and purifying recombinant protein are known in the art, for example, USSN 07/807,529. For example, CTB may be purified by affinity chromatography (Tayot et al. (1981) *Eur. J. Biochem.* 113:249-258), conjugated to cocaine or nicotine derivatives, and the conjugate may then be further purified. The purified CTB and the resulting conjugate are analyzed for purity and for maintenance of the pentameric structure of CTB. Techniques include SDS-PAGE, native PAGE, gel filtration chromatography, Western blotting, direct and GM1-capture ELISA, and competition ELISA with biotinylated CTB. Level of haptenation is measured by mass spectrometry, reverse phase HPLC and by analysis of the increase in UV absorbance resulting from the presence of the hapten. Both the solubility and the stability of the conjugate are optimized in preparation for full-scale formulation. Details of some of these analyses are given in the Examples.

Although the pentameric structure of CTB is a preferred carrier for practice of the present invention, and G_{M1} binding is an effective assay to determine that the pentameric form of CTB is present, the present invention is not limited to the use of the pentameric form of CTB. Other T cell epitope carriers are encompassed in the invention, as well as other forms of CTB (e.g., monomer, dimer, etc.) that may be manipulated for use in the invention. If a carrier other than the pentameric form of CTB is utilized, then one skilled in the art would use an appropriate assay to determine the presence and activity of the required carrier (e.g., the use of G_{M1} binding to determine the presence of the pentameric form of CTB).

Several conjugates produced according to the present invention include conjugates with analogs of cocaine and either BSA, HEL or CTB as the protein carrier. Six representative cocaine analogs are shown in Figure 3a. Of the six, PS-2, PS-4,

PS-5, PS-6, and PS-9 were conjugated with BSA or HEL, while PS-5 was also conjugated with CTB. (See Table 2 above). In addition, several conjugates according to the present invention include conjugates with analogs of nicotine.

5

In order to vary levels of hapteneation, alternative approaches are taken. In one embodiment the carrier is hapteneated with a multivalent cocaine or nicotine construct. This idea is based on the concept of multiple antigenic peptides (MAP) (Lu et al. *Mol. Immunol.*, 28:623-630 (1991)). In this system, multiple branched lysine residues are exploited to maximize hapten density and valency. The premise of this approach is that the immune response is enhanced if there are multiple copies of the hapten attached to the same peptide or protein molecule. Therefore, a multivalent hapten which needs to be attached to only one or two sites on the carrier CTB pentamer is prepared as set out herein. The core of such a multiple antigenic hapten is a branched polylysine core as suggested by Tam (Lu et al., *supra*). A chemically reactive handle is preserved by inclusion of a protected Cys residue. After cocaine or nicotine hapteneation of all available amino groups, the sulfhydryl of Cys is unmasked and made available for coupling to the protein with any of several bifunctional sulfhydryl/amino specific cross-linkers (Yoshitake et al. (1979) *Eur. J. Biochem.* 101:395-399. A number of dendimeric structures are used as a core.

Adjuvant

30 Any adjuvant which does not mask the effect of the carrier is considered useful in the cocaine and nicotine therapeutic vaccines of the present invention (see, Edelman (1980) *Rev. Infect. Dis.* 2:370-373). Initial experiments aimed at demonstrating the feasibility of a therapeutic vaccine against 35 cocaine addiction used the powerful adjuvant CFA (Figures 9a and c). However, CFA is not preferred in humans. A useful adjuvant

currently licensed for use in humans is alum, including aluminum hydroxide (Spectrum Chem. Mtg. Corp., New Brunswick, NJ) or aluminum phosphate (Spectrum). Typically, the vaccine is adsorbed onto the alum, which has very limited solubility.

5 Preliminary data in the murine model suggests that alum is capable of inducing a strong anti-cocaine antibody response (Figure 9b), and MF59 (Chiron, Emeryville, CA) or RIBI adjuvant is also suitable.

10 Effective immunization with CTB as the carrier protein does not require a powerful adjuvant. As shown in the Examples, high titer anti-cocaine antibody responses were induced by immunization with the CTB-cocaine conjugate either using alum as the adjuvant or in the absence of any added adjuvant. For 15 carriers other than CTB one skilled in the art would be capable of determining an appropriate adjuvant, if needed.

Excipients and Auxiliary Agents

20 Therapeutic compositions may optionally contain one or more pharmaceutically acceptable excipients including, but not limited to, sterile water, salt solutions such as saline, sodium phosphate, sodium chloride, alcohol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycol, gelatine, mannitol, 25 carbohydrates, magnesium stearate, viscous paraffin, fatty acid esters, hydroxy methyl cellulose, and buffer. Other suitable excipients may be used by those skilled in that art. The therapeutic composition may optionally comprising at least one auxiliary agent, for example, dispersion media, coatings, such 30 as lipids and liposomes, surfactants such as wetting agents and emulsifiers, lubricants, preservatives such as antibacterial agents and anti fungal agents, stabilizers and other agents well known to those skilled in the art. The composition of the present invention may also contain further adjuvants, agents 35 and/or inert pharmacologically acceptable excipients which may

be added to enhance the therapeutic properties of the drug or enable alternative modes of administration.

Highly purified hapten-carrier conjugates produced as discussed above may be formulated into therapeutic compositions of the invention suitable for human therapy. If a therapeutic composition of the invention is to be administered by injection (i.e., subcutaneous injection), then it is preferable that the highly purified hapten-carrier conjugate be soluble in aqueous solution at a pharmaceutically acceptable pH (that is, a range of about 4-9) such that the composition is fluid and easy administration exists. It is possible, however, to administer a composition wherein the highly purified hapten-carrier conjugate is in suspension in aqueous solution and such a suspension is within the scope of the present invention. The composition also optionally includes pharmaceutically acceptable excipients, adjuvant and auxiliary agents or supplementary active compounds. Depending upon the mode of administration, optional ingredients would ensure desirable properties of the therapeutic composition, for example, proper fluidity, prevention of action of undesirable microorganisms, enhanced bioavailability or prolonged absorption.

A therapeutic composition of the invention should be sterile, stable under conditions of manufacture, storage, distribution and use, and preserved against the contaminating action of microorganisms such as bacteria and fungi. A preferred means for manufacturing a therapeutic composition of the invention in order to maintain the integrity of the composition is to prepare the formulation of conjugate and pharmaceutically excipient such that the composition may be in the form of a lyophilized powder which is reconstituted in excipients or auxiliary agents, for example sterile water, just prior to use. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying, freeze-drying or spin drying which yields a powder of

the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The active compounds of this invention can be processed in accordance with conventional methods of galenic pharmacy to produce therapeutic compositions for administration to patients, e.g., mammals including humans. The preferred modes of administration are intranasal, intratracheal, oral, dermal, and/or injection. One particularly suitable combination of modes of administration comprises an initial injection with intranasal boosts.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampoules are convenient unit dosages. For enteral application, particularly suitable are tablets, dragees, liquids, suspensions, drops, suppositories, or capsules, which may include enteric coating. A syrup, elixir, or the like can be used wherein a sweetened vehicle is employed.

Sustained or directed release compositions can be formulated, e.g., liposomes or those wherein the active compound (conjugate) is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc. It is also possible to freeze-dry the new compounds and use the lyophilizates obtained, for example, for the preparation of products for injection.

For topical application, there are employed as nonsprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, ointments etc., which are, if desired, sterilized or mixed with auxiliary agent. For topical

application suitable are sprayable aerosol preparations wherein the active compound, preferably in combination with a suitable excipient or auxiliary agent, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous 5 propellant.

An antibody raised through the compositions and methods of the instant invention may have a molecular weight ranging from 150 KDa to 1,000 KDa. When the subject is exposed to free 10 cocaine or nicotine after vaccination with the optimized conjugate in the therapeutic composition, the free cocaine or nicotine is targeted by cocaine-specific or nicotine-specific antibody or antibodies. No changes in the form or structure of the drug are necessary for the antibody to recognize the drug in 15 vivo. While not intending to limit the present invention, it is believed that upon exposure of the vaccinated individual to cocaine or nicotine, the anti-drug antibodies will block the effects of cocaine and nicotine. At least three mechanisms are 20 believed to contribute to the blocking activity. First, antibodies are unable to cross the blood-brain barrier. Therefore, it is believed that cocaine or nicotine, when bound to the anti-cocaine or anti-nicotine antibody, will not cross 25 the blood-brain barrier and will not be able to exert its effect on dopamine transporters. Second, the antibody prevents the drug from binding to its receptor by simple steric blockade. This mechanism is expected to be operative in blocking some of 30 the non-CNS effects of the drugs (e.g. cardiac toxicity) and in the activity of antibodies against other drugs with non-CNS targets. Third, both cocaine and nicotine have relatively short 35 half-lives in vivo due to both enzymatic and non-enzymatic degradation, creating inactive metabolites. Cocaine and nicotine, in particular, are sufficiently small drugs so that it is very unlikely that they could cross-link antibodies, thus, it is highly unlikely that physiologically significant immune complex formation will occur for either of the drugs.

Still further embodiments of mucosal applications are used in the practice of the present invention. For example, copolymer microspheres are used to induce or enhance a mucosal immune response. These small, biodegradable microspheres encapsulate and protect the conjugate and facilitate uptake by the mucosal immune system. Although they are most widely used for oral immunization, they also have been reported to be effective with intranasal immunization (Walker (1994) *Vaccine* 12:387-399). Inert polymers such as poly(lactide-co-glycolide) (PLG) of 1-10 μm diameter are particularly useful in this regard (Holmgren et al. (1994) *Am. J. Trop. Med. Hyg.* 50:42-54; Serva (1994) *Science* 265:1522-1524).

In addition to the preferred conjugates, cross-immunization with different conjugates is carried out in order to minimize antibody cross-reactivity. Mice are primed with conjugates, more particularly PS-5 or PS-9 conjugates, and then boosted at day 14 with the reciprocal PS-9 or PS-5 conjugates coupled to the same carrier, BSA. Only the subset of antibody-secreting B cells that recognize both of the cocaine conjugates are maximally stimulated and expanded. It is believed that because the two conjugates differ in their point of attachment to the cocaine molecule, the specificity of the recognition increases. Specificity of the induced antisera is then confirmed by competition ELISA.

Still further, therapeutic compositions containing more than one conjugate stimulate polyclonal antibodies thereby enhancing antibody response upon subsequent challenge.

30

Dose

Neutralizing antibody responses against pathogens are known to last for years, and it should be possible to achieve a high-titer anti-cocaine or anti-nicotine antibody response that is maintained for at least a year. Based on values obtained with

conventional vaccines, it should be possible to achieve the concentrations of specific antibody required to neutralize cocaine plasma concentrations (1 - 10 μ M); the pharmacokinetic data in mice, described in the Examples, clearly demonstrates

5 that physiologically relevant neutralizing antibody concentrations can be achieved. Finally, the ability of maternal antibodies to cross the placenta in women addicted to cocaine and/or women who smoke, and thus protect the fetus, represents a further desirable effect of therapeutic cocaine

10 and/or nicotine vaccination. Optimizing therapy to be effective across a broad population is always challenging yet those skilled in the art use a careful understanding of various factors in determining the appropriate therapeutic dose. Further, antibody responses could be monitored using specific

15 ELISAs as set out in the Examples and other antibody based assays.

Genetic variation in elimination rates, interactions with other drugs, disease-induced alterations in elimination and

20 distribution, and other factors combine to yield a wide range of response to vaccine levels in patients given the same dose. Clinical indicators assist the titration of some drugs into the desired range, and no chemical determination is a substitute for careful observations of the response to treatment. Because

25 clearance, half-life accumulation, and steady state plasma levels are difficult to predict, the measurement of anti-drug-of-abuse antibody production is useful as a guide to the optimal dose. Each of the conjugates/carriers/adjuvants of the present invention is evaluated for the ability to induce an antibody

30 response that is best able to bind free cocaine or free nicotine in the circulation.

Further details about the effects of carriers and adjuvants on the induction of an antibody response are given in the

35 Examples.

Thus, it will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the specific conjugate being utilized, the particular compositions formulated, the mode of application, and the particular sites and organism being treated. For example, in one embodiment, the therapeutic composition containing a suitable carrier, is given first parenterally and boosted mucosally. As is discussed in more detail herein, this type of immunization with the optimal hapten and carrier combination is very effective in generating primarily IgG systemically and primarily IgA locally.

As set out in the Examples murine models have been used to demonstrate and measure different characteristics of the antibody response, including antibody titer, ability to recognize free cocaine, cocaine binding capacity, affinity for cocaine, specificity of the antibody response, antibody isotype, antibody tissue localization, and the physiological effects of the antibody following cocaine administration.

20

Antibody Titer

The first screen for vaccination is whether the conjugate of interest induces a high titer antibody response. Antibody titers are determined using an ELISA assay as described in the Examples below. Plates are coated with a cocaine-HEL conjugate, washed extensively, and incubated with varying dilutions of the test serum. The plates are again washed and developed with an enzyme-labelled anti-mouse IgG second antibody. Titers are defined as the reciprocal of the dilution of serum that gives 50% of the maximal response.

Antibody titer depends on both the concentration of antibody and on the antibody affinity. As detailed in the Examples, antisera with about 0.7 mg/ml cocaine-specific antibody of median affinity of about 2×10^{-8} M (or $5 \times 10^7 /M^{-1}$) had an

ELISA titer of 80,000. In estimating required antibody titer, both the concentration and the affinity of the antibodies are considered by those skilled in the art.

5 Although other methods of calculating appropriate antibody concentration are well known to those skilled in the art, without intending to limit the invention, one method of predicting anti-cocaine antibody concentration requirements is disclosed. Published peak plasma levels of cocaine in addicts 10 are in the range of 0.3-1.5 μ g/ml (Ambre et al. (1991) J. Anal. Tox. 15:17-20; Cone (1995) J. Anal. Tox. 19:159-478; and Cone et al. (1989) J. Anal. Tox. 13:65-68). Therefore, 0.075-0.375 mg/ml antibody is close to molar equivalence (The weight ratio of monoclonal antibody/cocaine = approximately 160,000/303= 15 approximately 500 but there are two binding sites on each antibody, so the molar ratio for binding site to cocaine is about 250). It is possible to achieve this level of antibody response with haptenated carrier, as demonstrated in the Examples. However, if a drug-of-abuse-specific dimeric 20 secreted-form IgA response is induced in the mucosa, as disclosed in at least one embodiment herein, the antibody concentration requirement on a molar basis is two-fold less relative to drug-of-abuse. It is not implied here that molar excess of antibody over drug-of-abuse is needed for successful 25 therapy.

In one therapeutic composition of the instant invention, cocaine-specific antibody (monoclonal antibody) blocked the effects of a molar excess of cocaine in a rat addiction model. 30 Rats were injected with 4 mg monoclonal antibody before infusion of cocaine (1 mg/kg; 300 μ g/rat). The measured concentration of monoclonal antibody in the rats was estimated to be about 80 μ g/ml. The antibody was at less than molar equivalence to the cocaine when compared either in the whole animal or in the 35 plasma.

Antibody affinity reflects the amount of antibody-drug complex at equilibrium with unbound antibody and unbound drug-of-abuse, thus:

$$K_{eq} = [\text{Ab+drug complex}] / [\text{Ab}] \times [\text{drug}]$$

5

where $[\text{Ab}]$ = molar concentration of unoccupied antibody binding sites; $[\text{drug}]$ = molar concentration of unbound drug; and $[\text{Ab+drug}]$ = molar concentration of antibody-drug complex.

10 For example, based on calculations, antibodies with affinity for cocaine above 10^{-6} M are mostly bound to cocaine and antibodies with affinities of about 10^{-7} M and are nearly all bound to cocaine at the expected antibody and cocaine plasma concentrations.

15

Ability to Recognize Free Cocaine

Once a conjugate is capable of inducing a high-titer serum antibody response, the serum also is tested for its ability to 20 recognize free cocaine in a competition ELISA as described in the Examples. An ELISA assay is set up using a suboptimal dilution of serum. Varying concentrations of free cocaine are added along with the antiserum, and the ELISA is developed as above. Data is expressed as the concentration of free cocaine 25 required to compete 50% of the antibody binding, an approximate measure of affinity. Lidocaine, among others, is used as a negative control in the competition experiments, and the cocaine-carrier conjugate that was used in the immunization is used as a positive control.

30

In addition to the competition ELISA assay, binding is assessed using radiolabelled cocaine. The data resulting from such assays can indicate if the immune serum is binding to free cocaine. Similar assays are used to determine binding of 35 nicotine specific antibodies. This is discussed in more detail in the Examples.

Specificity of Antibody Response

In order to be maximally effective at blocking the activity of cocaine, the induced antibodies must have minimal affinity for pharmacologically inactive metabolites of cocaine. Binding of antibodies to pharmacologically inactive metabolites of cocaine would reduce the potency of the vaccine. The primary inactive metabolites are ecgonine methyl ester and benzoyllecgonine each of which is commercially available. The specificity of the antisera for each of these metabolites is determined in a competition ELISA and by radiolabelled immunoassay. Furthermore, the effectiveness of the vaccine is increased if the induced antibodies bind to the pharmacologically active metabolites and derivatives of cocaine. The active metabolite of cocaine is norcocaine. The primary active derivative of cocaine is cocaethylene, formed by *in vivo* transesterification of cocaine, following co-administration of cocaine and ethanol. This is discussed in more detail in the Examples, below.

Additionally, interaction of the antibodies raised with other drugs used in addiction therapy and in other medical procedures should be minimized. In particular, cross reaction with drugs commonly prescribed to cocaine and poly drug abusers is avoided. While the unique nature of the cocaine tropane ring structure minimizes cross-reactivities, they can be readily tested in a competition ELISA. Indeed, lidocaine is used as a negative control in our competition ELISA. The following drugs are useful as co-treatments, buprenorphine, desipramine, naloxone, haloperidol, chlorproazine, mazindol and bromocriptine, as well as others that may become relevant.

Effect on Cocaine LD₅₀

Those conjugates and immunization protocols that are most effective at inducing high titer specific antibody responses are further evaluated for their ability to shift the cocaine LD₅₀. In these experiments, cocaine-immunized and control carrier-immunized mice are injected i.v. with cocaine at doses around the previously defined LD₅₀. Ten mice are used at each point, and the data is analyzed using a Cochran-Mantel-Haenzel Chi-squared test.

In addition, a failure time model is used to analyze the time-to-death induced by cocaine. The extent to which the anti-cocaine antibodies increase both (a) the dose of cocaine required for lethality and (b) the time-to-death are measures of efficacy in this model. These provide a rapid and rigorous test of the *in vivo* efficacy of the antibodies.

Observing the Physiological Effect on Humans

A person who seeks medical attention during an episode of abuse might present with a rapid pulse, an increased respiratory rate and an elevated body temperature. At high levels of overdose, the picture progresses to grand mal convulsions, markedly elevated blood pressure, and a very high body temperature, all of which can lead to cardio-vascular shock. In addition to blood levels, all these factors will be assessed and specific criteria will be established when administration of either active immunization with the vaccine or passive administration of antibodies to humans is contemplated.

When embodiments of the invention were tested on mice, immunization with a protein-cocaine conjugate induced an antibody response that shifts the LD₅₀ for cocaine (Figures 11a & b). It is hypothesized that the relatively small shift that was observed at very high doses of cocaine translates into a

more dramatic shift at lower cocaine concentrations; the dramatic effect of the anti-cocaine monoclonal antibody on cocaine self-administration is consistent with this hypothesis.

5 Without intending to limit the scope of the invention, the composition and methods of this invention will now be described in detail with reference to a preferred drug of abuse, cocaine, and specific embodiments.

10 Unless otherwise indicated in the Examples, female BALB/c mice of 2-3 months of age are used in these studies. These animals have a well defined reproducible response to the antigens under investigation. Animals are immunized either intramuscularly, subcutaneously, intratracheally, 15 intragastrically, or intranasally with a protein-cocaine conjugate either in saline, or on alum, or in CFA. Unless otherwise noted, BALB/c mice are immunized s.c. with 50 µg of test conjugate. After 14 days, mice are boosted with the same dose. In mice immunized using CFA, IFA was used for the 20 subsequent immunizations. Antibody responses in the serum are measured after an additional 14 days. Five mice are used per group and all sera are tested individually. CTB used in the following examples is commercially available, for example, from List or Sigma, or from SBL Vaccin.

25 Many of the following Examples specifically describe cocaine and an anti-cocaine antibody. These examples are, however, applicable to nicotine. For example, monitoring of the redistribution of nicotine (i.e., diminished amount in the 30 brain) is arrived at by injection of immunized mice with the tritium labelled nicotine (available from NEN), followed by decapitation at various time points. The effect of the anti-nicotine antibody on nicotine metabolism and clearance can be 35 analyzed either by TLC analysis of plasma taken from ³H nicotine injected mice or by HPLC.

It is to be understood that the example and embodiments described herein are for purposes of illustration only, and that various modification in light thereof will be suggested to persons skilled in the art. Accordingly, the following non-limiting Examples are offered for guidance in the practice of the instant invention.

EXAMPLE 1
Synthesis of PS-2

10 A solution of ecgonine methyl ester hydrochloride (50 mg, 0.21 mmol), diisopropylethylamine (80 μ l, 0.46 mmol) in DMF (3 ml) was treated with bromoacetyl bromide (22 μ l, 0.25 mmol) and heated at 40°C overnight. The solvents were removed under 15 reduced pressure and the residue purified by silica gel flash chromatography (9:1 chloroform:methanol as the eluent), furnishing the bromo compound (67 mg, 96%) as a pale yellow powder (3 β -(Bromoacetoxy)-8-methyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester).

20 To a solution of the bromo compound (17 mg, 0.053 mmol) in PBS (0.5 ml), thiolated BSA (15 mg) in PBS (0.5 ml) was added and stirring continued at ambient temperature for 3 days. The conjugate was purified by dialysis against PBS and then analyzed 25 by mass spectral analysis.

EXAMPLE 2
Synthesis of PS-4

30 To a solution of ecgonine methyl ester (32 mg, 0.16 mmol) in DMF (2 ml), triethylamine (22 μ l, 0.16 mmol), followed by succinic anhydride (16 mg, 0.16 mmol) was added and the solution heated at 35°C for 2 hours. The solvent was removed under 35 reduced pressure and the residue purified by silica gel flash chromatography (9:1 chloroform:methanol as the eluent). This

furnished the desired hemisuccinate (21 mg, 44%) as a white powder (3β -(Succinoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester).

To a solution of the hemisuccinate (2.4 mg, 7.69 μ mol) in distilled water (0.5 ml) at 0°C, EDC (1.5 mg, 7.69 μ mol) was added. After 10 minutes, BSA (2 mg in 0.5 ml PBS) and the solution allowed to warm to ambient temperature overnight. The conjugate was purified by dialysis against PBS and the degree of haptenation determined by mass spectral analysis.

EXAMPLE 3
Synthesis of PS-5

15 Method A

A solution of norcocaine hydrochloride (1 g, 3.07 mmol), triethylamine (0.86 ml, 6.14 mmol) in methylene chloride (20 ml) was treated with succinic anhydride (614 mg, 6.14 mmol) and the mixture heated at 45°C overnight. The solvents were removed under reduced pressure and the residue purified using silica gel flash chromatography (2:1 chloroform:methanol as the eluent). This gave succinylated norcocaine (1.0 g, 84%) as a thick syrup (3β -(Benzoyloxy)-8-succinoyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester).

To a solution of the acid (14 mg, 0.036 mmol) in distilled water (1 ml) at 0°C, EDC (10.4 mg, 0.055 mmol) was added. After 5 minutes a solution of BSA (14 mg) in PBS (1 ml) was added dropwise and the mixture allowed to warm to ambient temperature overnight. The conjugate was purified by dialysis against PBS and the degree of conjugation analyzed by mass spectral analysis.

Method B

To a solution of BSA (500 mg) in 0.2 M borate buffer (80 ml), succinic anhydride (270 mg, 2.70 mmol) in 1,4-dioxane (10 ml) was added in 200 μ l aliquots over 30 minutes. The pH was maintained at 9.3 by addition of 3 N sodium hydroxide solution. The solution was kept at ambient temperature for 18 hours, dialyzed against 0.01 M triethylamine and then lyophilized to yield 583 mg of a fluffy white powder. Mass spectral analysis of the product indicated 55 succinoyl groups per BSA molecule.

A solution of succinylated BSA (72 mg) in 0.1 M sodium bicarbonate buffer, pH 8.8 (15 ml) at 0°C was treated with EDC (88 mg, 0.46 mmol). After 5 minutes, norcocaine hydrochloride (100 mg, 0.31 mmol) was added and the solution allowed to warm to ambient temperature overnight. The conjugate solution was purified by dialysis against PBS and the degree of hapteneation determined by mass spectral analysis.

20

EXAMPLE 4
Synthesis of PS-6

To a solution of benzoyl ecgonine (276 mg, 0.96 mmol) in DMF (5 ml) at -10°C, borane-dimethylsulfide complex (1.0 M solution in methylene chloride; 1.0 ml, 1.01 mmol) was added dropwise. This was allowed to warm to ambient temperature overnight, after which the reaction was quenched by the addition of THF: water (1:1 ratio v/v) followed by stirring for a further 10 minutes. The solvents were removed under reduced pressure and the residue purified using silica gel flash chromatography (chloroform followed by methanol as eluents). This furnished the desired alcohol (246 mg, 93%) as a white powder (3 β -(Benzoyloxy)-2 β -(hydroxymethyl)-8-methyl-8-azabicyclo[3.2.1]octane).

35 To a solution of the alcohol (190 mg, 0.69 mmol) in DMF (5 ml), triethylamine (0.19 ml, 1.38 mmol) was added, followed by

succinic anhydride (138 mg, 1.38 mmol) and heated at 40°C overnight. The solvents were removed under reduced pressure and the residue purified using silica gel flash chromatography (1:1 chloroform: methanol as the eluent). This furnished the 5 hemisuccinate (123 mg, 48%) as a white powder (3β-(Benzoyloxy)-2β-(hydroxymethyl succinoyl)-8-methyl-8-azabicyclo[3.2.1]octane).

To a solution of the hemisuccinate (16 mg, 0.043 mmol) in 10 distilled water (0.5 ml) at 0°C, EDC (12 mg, 0.064 mmol) was added. After 5 minutes, BSA (16 mg) in PBS (0.5 ml) was added dropwise and the solution allowed to warm to ambient temperature overnight. The conjugate solution was purified by dialysis 15 against PBS and the degree of hapteneation determined by mass spectral analysis.

EXAMPLE 5
Synthesis of PS-9

To a solution of benzoyl ecgonine (10 mg, 0.035 mmol) in 20 distilled water (1.0 ml) at 0°C, EDC (10 mg, 0.052 mmol) was added. After 5 minutes BSA (10 mg) in PBS (0.5 ml) was added dropwise and the solution warmed to ambient temperature 25 overnight. The protein conjugate was purified by dialysis against PBS buffer. The degree of hapteneation was determined by mass spectral analysis.

EXAMPLE 6
Synthesis of CTB-PS-5

30

Method A

To a solution of succinylated norcocaine (2 mg, 5.14 µmol) in 35 DMF (0.1 ml), diisopropylethylamine (2 µl, 10.3 µmol) was added followed by HATU (2 mg, 5.40 µmol). After 10 minutes, the pale yellow solution was added dropwise to a solution of CTB (0.5 mg

in 0.9 ml of 10 mM borate buffer at pH 7.8) and shaken at ambient temperature for 1.5 hours. The pH of the conjugate solution was adjusted to pH 6.5 by the careful addition of 1 N HCl, followed by purification by dialysis against 20 mM sodium succinate, pH 6.5. The dialysate was filtered through a 0.2 μ m filter and the level of haptenation measured by mass spectral analysis or UV absorbance.

Method B

10 To a solution of succinylated norcocaine (2 mg, 5.14 μ mol) in DMF (0.1 ml), diisopropylethylamine (2 μ l, 10.3 μ mol) was added followed by HBTU (1.9 mg, 5.40 μ mol). After 10 minutes, the pale yellow solution was added dropwise to a solution of CTB 15 (0.5 mg in 0.9 ml of PBS buffer at pH 7.6) and shaken at ambient temperature for 1.5 hours. The pH of the conjugate solution was adjusted to pH 6.5 by the careful addition of 1 N HCl, followed by purification by dialysis against 20 mM sodium succinate, pH 6.5. The dialysate was filtered through a 0.2 μ m filter and the 20 level of haptenation measured by mass spectral analysis or UV absorbance.

Method C

25 To a solution of succinylated norcocaine (2 mg, 5.14 μ mol) in DMF (0.1 ml), diisopropylethylamine (2 μ l, 10.3 μ mol) was added followed by TNTU (1.9 mg, 5.40 μ mol). After 10 minutes, the pale yellow solution was added dropwise to a solution of CTB 30 (0.5 mg in 0.9 ml of PBS buffer at pH 7.6) and shaken at ambient temperature for 1.5 hours. The pH of the conjugate solution was adjusted to pH 6.5 by the careful addition of 1 N HCl, followed by purification by dialysis against 20 mM sodium succinate, pH 6.5. The dialysate was filtered through a 0.2 μ m filter and the 35 level of haptenation measured by mass spectral analysis or UV absorbance.

Method D

To a solution of succinylated norcocaine (2 mg, 5.14 μ mol) in DMF (0.1 ml), diisopropylethylamine (2 μ l, 10.3 μ mol) was added followed by TNTU (1.9 mg, 5.40 μ mol). After 10 minutes, the pale yellow solution was added dropwise to a solution of CTB (0.5 mg in 0.9 ml of 10 mM borate buffer at pH 7.8) and shaken at ambient temperature for 1.5 hours. The pH of the conjugate solution was adjusted to pH 6.5 by the careful addition of 1 N HCl, followed by purification by dialysis against 20 mM sodium succinate, pH 6.5. The dialysate was filtered through a 0.2 μ m filter and the level of haptenation measured by mass spectral analysis or UV absorbance.

Method E

To a solution of succinylated norcocaine (2 mg, 5.14 μ mol) in DMF (0.1 ml), diisopropylethylamine (2 μ l, 10.3 μ mol) was added followed by PyBroP (2.4 mg, 5.40 μ mol). After 10 minutes, the pale yellow solution was added dropwise to a solution of CTB (0.5 mg in 0.9 ml of PBS buffer at pH 7.6) and shaken at ambient temperature for 1.5 hours. The pH of the conjugate solution was adjusted to pH 6.5 by the careful addition of 1 N HCl, followed by purification by dialysis against 20 mM sodium succinate, pH 6.5. The dialysate was filtered through a 0.2 μ m filter and the level of haptenation measured by mass spectral analysis or UV absorbance.

Method F

To a solution of succinylated norcocaine (2 mg, 5.14 μ mol) in DMF (0.1 ml), diisopropylethylamine (2 μ l, 10.3 μ mol) was added followed by PyBroP (2.4 mg, 5.40 μ mol). After 10 minutes, the pale yellow solution was added dropwise to a solution of CTB (0.5 mg in 0.9 ml of 10 mM borate buffer at pH 7.8) and shaken at ambient temperature for 1.5 hours. The pH of the conjugate

solution was adjusted to pH 6.5 by the careful addition of 1 N HCl, followed by purification by dialysis against 20 mM sodium succinate, pH 6.5. The dialysate was filtered through a 0.2 μ m filter and the level of haptenation measured by mass spectral analysis or UV absorbance.

Method G

To a solution of succinylated norocaine (4 mg, 0.010 mmol) in dry DMF (0.22 ml), diisopropylethylamine (4 μ l, 0.023 mmol) was added, followed by addition of HATU (4 mg, 0.010 mmol). After 10 minutes at ambient temperature, the resulting pale yellow solution was added dropwise to rCTB (1 mg) in 0.1 M sodium borate, 0.15 M sodium chloride, pH 7.8 (2 ml). The resulting clear colorless solution was gently agitated for 1.5 hours at ambient temperature and then dialyzed against 20 mM sodium succinate, pH 6.5 at 4°C. The resulting conjugate was analyzed by GM1 capture ELISA, reverse phase HPLC and mass spectral analysis.

20

Method H

To a solution of succinylated norocaine (1 mg, 0.026 mmol) in dry DMF (0.22 ml), diisopropylethylamine (1 μ l, 0.0052 mmol) was added, followed by addition of HATU (1 mg, 0.0031 mmol). After 10 minutes at ambient temperature, the resulting pale yellow solution was added dropwise to rCTB (1 mg) in 0.1 M sodium borate, 0.15 M sodium chloride, pH 7.8 (2 ml). The resulting clear colorless solution was gently agitated for 1.5 hours at ambient temperature and then dialyzed against 20 mM sodium succinate, pH 6.5 at 4°C. The resulting conjugate was analyzed by GM1 capture ELISA, reverse phase HPLC and mass spectral analysis.

35

Method I

To a solution of succinylated norocaine (0.4 mg, 0.0010 mmol) in dry DMF (0.22 ml), diisopropylethylamine (0.4 μ l, 0.0023 mmol) was added, followed by addition of HATU (0.4 mg, 0.001 mmol). After 10 minutes at ambient temperature, the resulting pale yellow solution was added dropwise to rCTB (1 mg) in 0.1 M sodium borate, 0.15 M sodium chloride, pH 7.8 (2 ml). The resulting clear colorless solution was gently agitated for 1.5 hours at ambient temperature and then dialyzed against 20 mM sodium succinate, pH 6.5 at 4°C. The resulting conjugate was analyzed by GM1 capture ELISA, reverse phase HPLC and mass spectral analysis.

15 Method J

To a solution of succinylated norocaine (0.1 mg, 0.00010 mmol) in dry DMF (0.22 ml), diisopropylethylamine (0.1 μ l, 0.00052 mmol was added, followed by addition of HATU (0.1 mg, 0.0010 mmol). After 10 minutes at ambient temperature, the resulting pale yellow solution was added dropwise to rCTB (1 mg) in 0.1 M sodium borate, 0.15 M sodium chloride, pH 7.8 (2 ml). The resulting clear colorless solution was gently agitated for 1.5 hours at ambient temperature and then dialyzed against 20 mM sodium succinate, pH 6.5 at 4°C. The resulting conjugate was analyzed by GM1 capture ELISA, reverse phase HPLC and mass spectral analysis.

Method K

30 To a solution of succinylated norocaine (40 mg, 0.10 mmol) in dry DMF (0.22 ml), diisopropylethylamine (40 μ l, 0.23 mmol) was added, followed by addition of HATU (40 mg, 0.10 mmol). After 10 minutes at ambient temperature, the resulting pale yellow 35 solution was added dropwise via a pressure equalizing dropping funnel to rCTB (100 mg) in 0.1 M sodium borate, 0.15 M sodium

chloride, pH 8.5 (200 ml). The resulting clear colorless solution was gently agitated for 1.5 hours at ambient temperature and then diafiltered against 0.1 M ammonium bicarbonate, pH 8.5 at room temperature. Lyophilization afforded the buffer-free conjugate as a white powder, which was analyzed by GM1 capture ELISA, reverse phase HPLC and mass spectral analysis.

Method L

To a solution of succinylated norocaine (4 mg, 0.010 mmol) in dry DMF (0.44 ml), diisopropylethylamine (4 μ l, 0.023 mmol) was added, followed by addition of HATU (4 mg, 0.010 mmol). After 10 minutes at ambient temperature, the resulting pale yellow solution was added dropwise to rCTB (1 mg) in 0.1 M sodium borate, 0.15 M sodium chloride, pH 7.8 (2 ml). The resulting clear colorless solution was gently agitated for 1.5 hours at ambient temperature and then dialyzed against 20 mM sodium succinate, pH 6.5 at 4°C. The resulting conjugate was analyzed by GM1 capture ELISA, reverse phase HPLC and mass spectral analysis.

Method M

To a solution of succinylated norocaine (1 mg, 0.0026 mmol) in dry DMF (0.44 ml), diisopropylethylamine (1 μ l, 0.0052 mmol) was added, followed by addition of HATU (1 mg, 0.0031 mmol). After 10 minutes at ambient temperature, the resulting pale yellow solution was added dropwise to rCTB (1 mg) in 0.1 M sodium borate, 0.15 M sodium chloride, pH 7.8 (2 ml). The resulting clear colorless solution was gently agitated for 1.5 hours at ambient temperature and then dialyzed against 20 mM sodium succinate, pH 6.5 at 4°C. The resulting conjugate was analyzed by GM1 capture ELISA, reverse phase HPLC and mass spectral analysis.

EXAMPLE 7
Alternative Syntheses of CTB-PS-5

Method A

5 A solution of succinylated norcocaine (15 mg, 0.39 mol), thionyl chloride (28 μ l, 0.39 mmol) in DMF (250 μ l) was stirred at ambient temperature for 2 hours. After the reaction was deemed complete (by TLC analysis), the solvents were removed
10 under reduced pressure and the resulting chloro derivative (3 β -(Benzoyloxy)-8-chlorosuccinoyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester) taken through to the next step without further purification.

15 The chloro derivative (16 mg, 0.04 mmol) was dissolved in DMF (100 μ l) and added dropwise to a solution of CTB (0.38 mg/ml in 3 ml PBS). The resulting mixture was kept at ambient temperature overnight, dialyzed against PBS and the degree of haptenation determined by mass spectral analysis.

20

Method B

25 To a solution of succinylated norcocaine (100 mg, 0.26 mmol) in DMF (5 ml), DCC (64 mg, 0.31 mmol) was added. After 10 minutes, 4-hydroxy-3-nitrobenzene sulfonic acid sodium salt (74 mg, 0.31 mmol) was added and the resulting yellow solution kept at ambient temperature for 4 days. The resulting suspension was filtered under reduced pressure and the filtrate added to cold diethyl ether (10 ml) with vigorous stirring. Hexane (5 ml) 30 added and after complete precipitation of a yellow oil, the colorless supernatant was decanted off. This process was repeated and the oil dried overnight under reduced pressure, furnishing the desired ester (157 mg) (3 β -(Benzoyloxy)-8-(2-nitro-4-sulfophenyl ester)succinoyl-8-azabicyclo[3.2.1]octane-35 2 β -carboxylic acid methyl ester) which was taken through to the next stage without further purification.

The ester (5 mg, 8.16 μ mol) was dissolved in DMF (100 μ l) and added dropwise to CTB (1 mg in 2 ml PBS) at 4°C and then warmed to ambient temperature. After 3 hours the conjugate solution was purified by dialysis against PBS and the degree of haptenation determined by mass spectral analysis.

Method C

10 To a solution of succinylated norcocaine (108 mg, 0.28 mmol) in DMF (5 ml) at 0°C, NMM (37 μ l, 0.33 mmol) followed by ethyl chlorofomate (32 μ l, 0.33 mmol) were added. After 10 minutes, N-hydroxysuccinimide (38 mg, 0.33 mol) was added and the solution warmed to ambient temperature over 18 hours. The 15 solvents were removed under reduced pressure and the residue recrystallized from isopropanol/diethyl ether to furnish the N-oxysuccinimidyl ester (113 mg, 84%) as a white powder (3 β -(Benzoyloxy)-8-(N-oxysuccinimidoyl)succinoyl-8-azabicyclo[3.2.1]octane-2 β -carboxylic acid methyl ester).

20 A solution of the ester (2 mg, 4.11 μ mol) in DMF (100 μ l) was added dropwise to a solution of CTB (1 mg in 2 ml PBS). After 3 days the conjugate solution was purified by dialysis against PBS and the degree of haptenation determined by mass spectral 25 analysis.

EXAMPLE 8

Synthesis of a Conjugate with an Extended Spacer

30 To a solution of norcocaine hydrochloride (50 mg, 0.15 mmol) in DMF (1 ml), diisopropylethylamine (27 μ l, 0.31 mmol) was added. After 5 minutes the solution was cooled to 0°C and added dropwise to a solution of adipoyl chloride (44 μ l, 0.080 mmol) in DMF (100 μ l) at 0°C. After 2 hours the solution was added 35 dropwise to a solution of CTB (1 mg in 2 ml PBS) at 0°C and warmed to ambient temperature overnight. The conjugate solution

was purified by dialysis against PBS and the degree of haptenation determined by mass spectral analysis.

EXAMPLE 9

Conjugation of Succinylated Norcocaine with MAP

MAP resin (Novabiochem USA, La Jolla, CA) (substitution level: 0.48 mmol/g; 50 mg, 0.023 mmol) was pre-swollen in DMF (5 ml). The solvent was decanted and the resin treated with a 10 solution of 20% piperidine in DMF (5 ml), agitated for 15 minutes and the solvents removed by decanting. The resin was washed sequentially with DMF (5 ml), methanol (5 ml) and DMF (5 ml). A solution of succinylated norcocaine (18 mg, 0.046 mmol) in DMF (1 ml) was treated with a mixture of HOBT/DMF/HATU (0.5 M 15 freshly prepared solution in DMF; 92 μ l, 0.046 mmol) and after 5 minutes, this was agitated overnight after which the reaction was deemed to be >90% complete by the Kaiser-Ninhydrin test. The solvents were decanted off and the resin beads washed 20 exhaustively with methanol, followed by drying under a stream of argon. The derivatized MAP was cleaved by suspending the resin in 2.5% phenol/TFA/EDT (5 ml) and agitating for 1 hour, filtered, washed with TFA (4 x 4 ml) and the solvents removed under reduced pressure. The crude peptide was triturated with cold diethyl ether, centrifuged for 5 minutes at 5000 rpm and 25 the process repeated. The pellet was dissolved in water and lyophilized to give 1 mg of crude peptide.

EXAMPLE 10

Synthesis of (N-succinamidyl-cocaine)₈ -MAP Protein Conjugate

30 Synthesis of the non-hapten portion (MAP core) of the poly-haptenated MAP is carried out by manual peptide synthesis as described by Tam et al (U.S. Patent 5,229,490). Amino groups are protected by the Boc (t-butyloxycarbonyl) function and the 35 sulphhydryl group of Cys is protected as its 3-nitro-2-pyridylsulfenyl (Npys) derivative. After assembly on the resin

and removal of Boc protecting groups with TFA as described by Tam (*supra.*), the MAP core is cleaved from the resin by HF cleavage leaving the Npys group intact. Crude MAP core is taken up in 7 M guanidine hydrochloride containing 0.2 M HOAc and subjected to gel permeation chromatography in 0.2 M HOAc on Sephadex G-10 to remove any remaining low molecular byproducts generated by the HF cleavage. The MAP core is lyophilized from 0.2 M HOAc. (N-succinimidyl-norcocaine)₈-MAP is prepared according as described in Example 9.

Prior to coupling to activated protein the thiol group is exposed by treatment with a molar equivalent of tris-(2-carboxyethyl) phosphine hydrochloride (TCEP). Activated protein carrier is dissolved at 5 mg/ml in 0.2 M sodium bicarbonate buffer at room temperature. To this solution is added a 2-fold molar excess of (N-succinimidyl-norcocaine)₈-MAP at 5 mg/ml. The reaction is allowed to proceed for 20 hours at room temperature and then dialyzed overnight against 0.2 M HOAc and lyophilized.

20

EXAMPLE 11

Testing the Induction of Cocaine Specific Antibody Response

In order to induce an antibody response against a small molecule or hapten, such as cocaine, it is necessary to link it to a T cell epitope-containing carrier, e.g., a protein carrier. The carrier is recognized by T cells which provide help to the cocaine-specific B cells for initiation and maintenance of sustained antibody production. In this example, the carrier used was BSA, a protein which has 36 lysine residues that are exposed and available for conjugation. A panel of structurally distinct cocaine-protein conjugates were produced that were linked through different regions of the cocaine molecule (Figures 1a, 1b, 2a, 2b). A set of conjugates was synthesized because the cocaine molecule is physically altered and differently oriented during the conjugation process to the

carrier. Since any given cocaine conjugate may induce antibodies which recognize the conjugate only, and not the free hapten (cocaine) itself, screening was performed.

5 Mice were immunized with 50 µg of cocaine-BSA conjugate PS-5.1 and PS-5.6 (Figures 9a and b) or with PS-9.1 (Figure 9c) i.p. either with CFA (Figures 9a and 9c) or with alum (Figure 9b). Mice were boosted one time and then bled. The mice immunized with cocaine-BSA conjugate PS-5.1 were boosted with 10 cocaine-BSA conjugate PS-5.6. Sera were tested in an ELISA assay using plates coated with PS-5 (conjugated to HEL) or PS-9 (conjugated to HEL) as appropriate. The responses of 5 individual mice per group are shown. These data demonstrate that the cocaine-BSA conjugates are able to induce high titer 15 antibody responses.

EXAMPLE 12
Recognition of Free Cocaine

20 To directly determine whether the induced antibodies were capable of recognizing the free cocaine molecule, a competition ELISA was established. Plates were coated with appropriate free cocaine-HEL conjugate and incubated with the antisera at a 1:2000 dilution in the presence of varying concentrations of 25 free cocaine as competition. When PS-5.6-BSA was used as the immunogen, the majority of the antibody response was effectively competed by free cocaine (Figures 10a and b). In this set of sera from ten mice, (each line on the graph in Figure 10a indicates a different mouse) one was less effective in the 30 competition assay (open squares and dotted line), and this mouse was not used in the LD₅₀ experiments described herein. The PS-9.2-BSA conjugate also induces cocaine-specific antibodies. These data demonstrated that cocaine-carrier conjugates can be synthesized which induce high-titer, cocaine-specific antibody 35 responses that should be capable of neutralizing cocaine *in vivo*.

EXAMPLE 13

Ability of Vaccination to Protect Against Cocaine Toxicity

5 The present invention discloses a cocaine-protein conjugate that induced an anti-cocaine antibody response in a mouse model. These anti-cocaine antibodies neutralized cocaine *in vivo*, significantly shifting the dose of cocaine required to induce a lethal response in mice.

10 The efficacy of therapeutic vaccination against cocaine was assessed by determining the lethal dose of cocaine (LD_{50}) in immunized and naive animals. The prediction was that a strong cocaine-specific antibody response should bind sufficient 15 quantities of cocaine to prevent the rapid cardiac, respiratory, and neurological effects of cocaine, thus increasing the LD_{50} of cocaine in the immunized mice. Sixty BALB/c mice were immunized with 50 μ g PS-5.4-BSA in CFA and boosted only once with the same conjugate in IFA. Each of the mice was bled at day 34 and 20 serum antibody titers and competition with cocaine were assessed. Forty-eight mice were chosen for the experiment, with average titers of 18,700, all of which displayed competition with free cocaine. For the LD_{50} experiment, 4-6 mice were used per group and each group was carefully matched for antibody 25 titer and apparent affinity for free cocaine.

As shown in Figure 11, the LD_{50} for cocaine in naive BALB/c mice was 3 mg/kg when the drug was given intravenously (i.v., Figure 11b) and 20 mg/kg when given intraperitoneally (i.p., Figure 11a). Immunization of mice with the cocaine-protein conjugate changed the LD_{50} significantly. The doses required for half-maximal toxicity were 4.5 mg/kg and 35 mg/kg for the i.v. and i.p. doses, respectively. These doses were significantly different from the value obtained in the naive mice ($p = 0.048$ for i.v. and $p = 0.014$ for i.p., Cochran-Mantel-Haenszel Chi-squared test). The almost two-fold protection of

acute high dose toxicity by cocaine vaccination compares favorably with some drugs affecting cocaine pharmacology. For example, the NMDA antagonist MK-801 increased the LD_{50} 1.3-fold and 1.4-fold when combined with propanolol (Itzhak et al. (1992) 5 *J. Pharmacol. Exp. Therap.* 262:464-467). In addition, vaccination significantly prolonged the time to death from an average of 3.2 min to 5.4 min. for i.v. administration ($p = 0.007$, Wilcoxon 2-sample test) and from 4.0 min to 8.5 min. for i.p. administration ($p = 0.0003$). This study demonstrates that 10 the antibody affected the *in vivo* physiological response to high dose cocaine.

EXAMPLE 14

Discrimination of Cocaine from Saline in Rat Model

15 To demonstrate the stability and reproducibility of this system, 8 rats are trained to discriminate i.p. injections of 10 mg/kg cocaine from saline using a 2-lever procedure (Kantak et al. (1995) *J. Pharmacol. Exp. Therap.* 274:657-665). After 20 cocaine injections are given, the animals are required to press one of the levers (drug-appropriate lever) 10 times (FR 10) to obtain a food pellet; upon saline injections, they are required to press the other lever (saline-appropriate lever) 10 times to obtain a food pellet. When animals have learned to discriminate 25 cocaine from saline, at least 90% of the total responses are made on the appropriate lever for several consecutive days. In order to incorporate a cumulative dosing procedure during later substitution test sessions, training sessions are made up of multiple components, each lasting for 10 min or until 10 FRs are 30 completed, whichever occurred first.

Following training, substitution test sessions with different doses of cocaine (0.3 - 17.8 mg/kg) are conducted twice weekly, with training sessions on intervening days. Drug substitution 35 test sessions consisted of four 10 min components, each preceded by a 15 min time-out period. During substitution tests,

completion of 10 responses on either lever produce a food pellet. Incremental doses of cocaine are injected at the beginning of each of the 4 time-out periods. Overlapping ranges of cumulative doses are studied on different test days, 5 permitting a seven-point cumulative dose-response curve to be determined in a single week.

In substitution tests, cocaine engendered dose-related increases in the percentage of cocaine-appropriate responses, which result 10 in full substitution (> 90% cocaine-appropriate responses) for all subjects after administration of doses that are at least the level of the training dose. Each data point is based on 2-3 determinations in individual subjects. The $ED_{50} \pm 95\% C.I.$ for cocaine-appropriate responses is $2.14 \pm 0.20 \text{ mg/kg}$, which 15 compares favorably to the value obtained in rats trained to discriminate injections of 10 mg/kg cocaine using single component and single dosing procedures ($2.6 \pm 0.29 \text{ mg/kg}$; (Kantak et al. (1994) *J. Pharmacol. Exp. Ther.* (under review)).

20

EXAMPLE 15

Assays to Detect the Function Activity of CTB

To test the functional activity of CTB alone, two assays were developed. First, binding of CTB to cells was measured using 25 flow cytometry. Cells were incubated with CTB, followed by a commercial anti-CTB goat antiserum and a fluorescein isothiocyanate (FITC)-labelled anti-goat secondary antibody (Figure 13). Native pentameric CTB bound to the cells, causing a dramatic shift in fluorescence intensity. Monomeric CTB was 30 unable to bind to cells in this assay. Second, an ELISA was set up to measure the ability of the CTB to bind to ganglioside GM1. ELISA plates were coated with GM1-ganglioside and incubated with varying concentrations of CTB. Binding was detected using an anti-CTB antibody (or saline as a control) followed by enzyme- 35 labelled second antibody and development with substrate. This assay provided a quantitative and extremely sensitive measure of

the ability of pentameric CTB to bind to GM1 gangliosides. These assays are used to monitor the functional activity of recombinant and hapteneated CTB conjugates prior to experiments in vivo. Similarly, Figure 14a shows that conjugation does not affect the ability of CTB-specific antibodies to recognize the conjugate. Figure 14b shows that the conjugated CTB molecules which are able to bind GM1 can also be bound by cocaine-specific antibodies, demonstrating the retention of CTB activity by hapteneated CTB.

10

EXAMPLE 16

Self-Administration Model of Addiction and Effect of Vaccine

In rats, the reinforcing stimulus properties of cocaine can be studied reliably using intravenous self-administration procedures. This is a direct model of addiction and drug self-administration behavior in animal subjects which positively correlates with abuse of that drug by human subjects. To examine the effect of the therapeutic vaccine, adult male rats (Wistar, approximately 300 g) are implanted with a chronic jugular vein catheter using the general procedures described by Weeks (*Meth. Psychobiol.* (1972) 2:115-168) and as adapted by Kantak et al. (Kantak et al. (1990) *Pharm. Biochem. Behavior* 36:9-12; (Kantak et al. (1991) *Psychopharmacol.* 104:527-535; and 25 Kantak et al. (1992) *Pharmacol. Biochem. Behav.* 41:415-423). All animals are housed individually and maintained at 80%-85% of their free feeding body weights to facilitate comparison with the drug discrimination experiments. One week following surgery, 1.0 mg/kg/infusion of cocaine is available as the 30 training dose in daily 2 hr sessions. Rats typically self-infuse a cumulative dose of 10 mg/kg each hour. During the initial phase of training, each lever press results in drug delivery. The required number of responses to self-infuse cocaine is increased gradually to 5 (FR 5) and then the FR 5:FI 35 5 min schedule of drug delivery is introduced. Following stable responding for at least 5 days, a baseline cocaine dose-response

curve (0.1, 0.3, 0.56, 1.0 and 3.0 mg/kg/infusion) is determined. Each dose of cocaine, as well as saline, is examined for a block of at least 5 sessions and until no systematic upward or downward trends in responding are observed.

5 Data is expressed as mean response rates over the last two days of each block of sessions.

Following determination of the baseline cocaine dose-response curve in 30 rats, half the rats are immunized with the optimal cocaine-carrier conjugate and the other half are immunized with carrier alone. Self-administration sessions are discontinued until significant anti-cocaine antibody titers are achieved, which should take 4-6 weeks. Rats are bled from the tail vein to ensure that all rats have comparable titers of cocaine-specific antibodies. Following immunization, the rats are tested for their ability to respond to cocaine. Rats will have access to varying doses of cocaine (0.3-3.0 mg/kg/infusion), or to saline, in 5-day blocks. Control rats immunized with carrier alone quickly return to the baseline pattern of cocaine self-administration.

To determine if anti-cocaine antibody blocks the reinforcing effects of cocaine, doses of cocaine up to 30 mg/kg/infusion are examined to determine how much protection the antibody affords.

25 If the anti-cocaine antibody partially blocks cocaine, the rats require much larger doses of cocaine to achieve the desired physiological effect and responses maintained by cocaine are reinstated with a rightward shift in the cocaine dose-response curve. If the polyclonal cocaine antibody completely blocks

30 doses of cocaine up to 30 mg/kg/infusion, then responding which is maintained by cocaine is not reinstated and cocaine self-administration extinguishes, with the cocaine dose-response curve remaining flat at near-zero saline-like levels.

Cocaine self-administration can also be inhibited by passively administered anti-cocaine antibody. Monoclonal anti-cocaine antibody or control antibody was administered to separate groups of rats. Animals that had been previously stabilized on a FR5: F15 schedule of cocaine administration extinguished their self-administration of cocaine if passively treated with anti-cocaine antibodies. Rats treated with control antibody maintained their cocaine self-administration responses.

EXAMPLE 17

Co-Treatment with Other Drugs

Screening is done to determine whether pharmacotherapy with buprenorphine, mazindol, and/or desipramine will enhance the 15 activity of the therapeutic vaccine. Treatment with buprenorphine, mazindol, and/or desipramine are expected to be compatible. It is possible that the therapeutic agents could be immunosuppressive, thus inhibiting the induction of a high titer anti-cocaine antibody response. To address this possibility, 20 rats are immunized with the cocaine-carrier conjugate in the presence or absence of buprenorphine or desipramine and the antibody titer is measured at varying times. A drug which is found to be significantly immunosuppressive will be eliminated 25 as an incompatible therapy. This screening test is used for any drug for which co-treatment is considered.

If no immunosuppression is seen, further screening is carried out to determine if the two approaches synergize. Following training, immunization and testing, rats are further evaluated in the two models in the presence of the drugs. Rats will receive drugs before sessions with different doses of cocaine. Initial experiments with control carrier-immunized rats are performed to choose a dose of drug that does not completely extinguish behavior in the self-administration or drug discrimination systems; it is estimated that this dose is approximately 24 µg/kg/day (-)-buprenorphine 20 µg/kg/day

mazindol, or 2 mg/kg desipramine. Data is evaluated to determine whether the action of the therapeutic vaccine is additive with the treatment with buprenorphine or desipramine.

EXAMPLE 18

5 Induction of Mucosal Response

The B subunit of cholera toxin (CTB) has been shown in many systems to retain the activity of intact cholera toxin, 10 including the induction of a mucosal antibody response. Therefore, this carrier should induce a strong anti-cocaine or anti-nicotine IgA antibody response. In addition, oral priming should induce a strong systemic IgG antibody response 15. An effective way to prime an immune response in the respiratory tract is to deliver antigen directly to those sites. The antigen is administered in saline, with CTB acting as its own adjuvant. To confirm the ability of CTB to prime by administration at a mucosal IgA surface, initial experiments are 20 conducted with carrier alone. Mice are primed with 50 µg of the CTB or cocaine-CTB or nicotine-CTB conjugate by three routes: orally, nasally or intratracheally. For oral administration of mice, 250 µg of either cocaine-CTB or nicotine-CTB conjugate or CTB alone is applied intragastrically, 25 or directly to the stomach, through the use of a blunt 23G needle. Fourteen days after priming, the mice are boosted using the same protocol. Nasal administration is a simple and common route of priming. Antigen is applied to each nostril of a lightly anesthetized mouse, for a total volume of 50 µl per 30 mouse. Fourteen days after priming, the mice are boosted using the same protocol. Nasal administration is adaptable readily to human application as a nasal spray. Nasal vaccination has been used successfully with live influenza vaccines (Walker et al. 35 (1994) Vaccine 12:387-399).

Intratracheal immunization directly applies the antigen to the lower respiratory tract, thereby enhancing immunity in the lungs. Mice are anesthetized with a cocktail of ketamine and xylazine. The animals are mounted on an apparatus that holds their mouth open and exposes the trachea; the trachea is visualized with a fiberoptic light probe. A blunt 23 gauge needle is used to deliver 50 μ l of solution into the lungs. Fourteen days after priming, the mice are boosted using the same protocol.

10 Animals are sacrificed by CO_2 asphyxiation at varying time points after boost (14, 21, or 28 days) and nasal and bronchoalveolar lavage fluids are collected and assayed for IgA specific for the administered conjugate. Nasal wash fluid is 15 obtained by washing the nasal cavity four times with a total of 1 ml PBS as described (Tamura et al. (1989) *Vaccine* 7:257-262). Bronchoalveolar lavage fluid is obtained by surgically exposing the trachea, injecting 0.5 ml PBS into the lungs, and rinsing three times as described (Nedrud et al. (1987) *J. Immunol.* 20 139:3484-3492). Following centrifugation to remove cells, samples are assayed for antigen-specific IgA by ELISA using an IgA-specific second antibody. Cocaine-specific or nicotine-specific IgG is measured in the nasal and lung washes, as it has been reported that IgG is frequently both detectable and 25 important in the lung (Cahill et al. (1993) *FEMS Microbiol. Lett.* 107:211-216).

The oral immunization route is evaluated for its ability to generate cocaine-specific or nicotine-specific IgA in intestinal 30 washes and is compared with other routes for its ability to generate serum Ig specific for cocaine or nicotine. Oral administration is particularly preferred in humans due to ease of administration. The intranasal and intratracheal routes of administration are compared directly for their ability to induce 35 an IgA response in both the lung or nasal lavage fluid. Whichever route is found to be most potent, it is preferred and

used for the remaining experiments. If the two routes are of comparable efficacy, nasal immunization is preferred because of its simplicity.

5 For maximal protection against cocaine or nicotine, systemic IgG and mucosal IgA responses may both be maximized. Therefore, both a systemic injection with the cocaine-CTB or nicotine-CTB conjugate in alum (or some other adjuvant) and a mucosal challenge with the conjugate are preferred to effectively prime 10 both compartments. Three groups are compared. First, mice are primed systemically, followed by a mucosal challenge after 14 days. Second, the mice are primed mucosally, followed by a systemic challenge after 14 days. Third, they are primed both systemically and mucosally at the same time, followed by an 15 identical boost after 14 days. Control mice are primed only mucosally or only systemically. In each case, efficacy in challenge is determined by measurement of both IgG and IgA anti-cocaine antibody titers.

20 As an initial measure of the *in vivo* efficacy of mucosal anti-cocaine or anti-nicotine antibodies, the change in drug pharmacokinetics is measured for mucosally administered cocaine or nicotine, respectively.

25

EXAMPLE 19

Immunogenicity of Cocaine-CTB Conjugates

A. Definition of Dose Required for Immunogenicity

30 The immunogenicity of cocaine-CTB conjugates was determined by immunization of rodents with cocaine-CTB, boosting where appropriate, and assessing antibody levels at varying times. Antibody levels were measured in an antigen-specific ELISA. Antibody titers were determined as the reciprocals of the serum 35 dilution giving 50% of the maximal response in the ELISA and are

expressed as the geometric means of the results from 5 or more mice.

To determine the range of antigen dose required to induce an anti-cocaine antibody response, mice were immunized either subcutaneously or intramuscularly with varying doses of cocaine-CTB PS-5.53. Animals were boosted on days 23 and 59 and bled on day 71. Doses of 3, 10, and 30 µg given intramuscularly induced titers of cocaine-specific IgG of 18429, 29013, and 22957, respectively. Using s.c. immunization, the same doses induced specific antibody titers of 10097, 15136, and 21169. These data demonstrate that cocaine-CTB can be effectively used in the range of 3-30 µg and greater and lower doses are expected to be effective. Similar doses are also effective for use in rats. Those skilled in the art use this data to identify optimal human doses, which are usually comparable.

B. Immunization on Mucosal Surfaces

To generate optimal antibody responses in mucosal secretions, it is usually necessary to prime at a mucosal surface. To determine whether CTB would be a useful carrier protein for the induction of a mucosal antibody response, mice were immunized intranasally or intratracheally. The methods for intranasal and intratracheal immunization are described in Example 18. Intranasal immunization with cocaine-CTB induced significant levels of circulating cocaine-specific IgG, although the titers were lower than those seen following subcutaneous or intramuscular immunization. As with the routes of administration described in Part A of this example, doses of cocaine-CTB of 3-30 µg all induced significant levels of cocaine-specific antibody. Simultaneous immunization by subcutaneous and intranasal routes induced antibody titers indistinguishable from those induced by the subcutaneous route alone. The feasibility of the intratracheal route of immunization was assessed by immunization with CTB alone. This

route was also found to induce antigen-specific IgG in the serum (CTB-specific in this case). These data demonstrate that CTB is capable of inducing a systemic antigen-specific IgG response following immunization at a mucosal surface in the absence of any added adjuvant.

C. Induction of Cocaine-Specific Antibodies in Mucosal Secretions

10 To maximize protection against the addictive properties of cocaine, it is desirable to optimize the levels of cocaine-specific antibody at the sites of cocaine application (e.g. nasal and lung mucosa) as well as in the blood. Mice were immunized intranasally or subcutaneously with 10 µg cocaine-CTB 15 and were boosted using the same protocol on days 27 and 61. Following sacrifice on day 78, bronchial and nasal washes were collected as described in the Examples and assayed for cocaine-specific IgA and IgG. Anti-cocaine antibodies were detectable in both the nasal and bronchial washes using both immunization 20 regimens. Intranasal immunization induced higher levels of antigen-specific IgA, while both routes were comparable at inducing anti-cocaine IgG responses in the mucosal secretions. The intranasal route of administration was also found to be the most effective route for the induction of antigen-specific IgA 25 in the serum. Intratracheal immunization with CTB also induced CTB-specific IgA and IgG in the respiratory secretions. These data demonstrate that CTB is an effective carrier protein for the induction of an antigen-specific antibody response in the respiratory tract.

30

D. Use of Alum as Adjuvant for Immunization

The use of adjuvant is often beneficial in immunization protocols. To assess the contribution of alum to the immune 35 response, mice were immunized with 10 µg cocaine-CTB PS-5.53 intraperitoneally in saline or adsorbed onto alum. The mice

were boosted at day 27 using the same protocol. For both groups of animals, high levels of cocaine-specific antibodies were detected by day 43 (titer of 14687 without alum and 16775 with alum). Immunization with cocaine-CTB adsorbed onto alum has also been shown to be effective with a subcutaneous or intramuscular route of administration. Therefore, the use of alum is acceptable with this antigen.

The addition of alum adjuvant can increase the immune response to injected proteins. Obtaining sufficient antibody titers requires testing the contribution of alum to the antibody response after injection of drug-carrier conjugates. To assess the contribution of alum mice were immunized with 10ug cocaine-rCTB PS-5.189, where the CTB was recombinantly expressed in bacteria. The mice were injected intramuscularly in saline or absorbed onto alum and again on day 14. For these lots of cocaine-CTB conjugates the addition of alum is required in order to generate anti-cocaine antibodies as detected by ELISA.

20 E. Duration of Antibody Responses

To determine whether antibody responses induced with cocaine-CTB PS-5.8 are long-lasting, serum antibody levels were monitored as a function of time. The animals described in section D of this Example were monitored out to day 128 after immunization. At that time point, antibody titers remained high, dropping approximately 2-fold from the peak at day 43. These data demonstrate that anti-cocaine antibody responses to cocaine-CTB conjugate are long-lasting.

25 F. Relative Levels of Anti-Hapten and Anti-Carrier Antibody Response

30 Immunization with cocaine-CTB induces an antibody response against both the hapten (cocaine) and the carrier (CTB). CTB is

a very powerful immunogen and it is possible that the anti-CTB response could dominate, preventing the anti-cocaine response from reaching very high titers. To determine whether it was possible to differentially regulate the anti-cocaine and anti-
5 CTB antibody response to CTB by changing the immunization regimen, the following nonlimiting test was performed. Mice were intramuscularly immunized with 30 µg cocaine-CTB and monitored for antibody response. This immunization induced both anti-cocaine and anti-CTB antibodies with the relative ratio of 10 the serum IgG titers being 0.04. In contrast, a ratio of 0.2 was seen when the mice were immunized with 3 µg cocaine-CTB. These doses of 3 µg and 30 µg produce similar titers of 18429 and 22957, respectively. It is likely that the ratio of anti-cocaine to anti-CTB antibodies will also be affected by other 15 parameters of the immunization regimen as well as by properties of the conjugate itself, such as level of haptenation.

EXAMPLE 20

Direct Binding of Cocaine by Antibodies From Immunized Mice

20 The ability of the antibodies to bind free cocaine can be assessed using radiolabelled cocaine. ³H-Cocaine (1 µCi) was incubated with serum from normal mice (0.05 ml), with serum from mice immunized with a PS-5.4 conjugate (conjugated with BSA) 25 (0.05 ml, pool of serum from 10 mice) or with commercially available anti-cocaine monoclonal antibodies (mixture of two different antibodies, 2 µg of each) (see Figure 10b). Beads coated with protein G were included in the incubation to bind to the Fc portion of antibody molecules. After 2 hours, the beads 30 were pelleted by centrifugation, washed three times with cold PBS and counted in a scintillation counter. The data in Figure 10b represent the mean and standard deviations of duplicate samples. These data clearly show that the immune serum is able to bind free cocaine with an affinity sufficiently high to 35 permit the bound cocaine to be precipitated and washed. This is

evidence that these antibodies will be able to bind and neutralize cocaine in the circulation of cocaine addicts.

EXAMPLE 21

5 Specificity of Cocaine-Specific Antibodies

To further analyze the specificity of the cocaine specific antibodies induced by the cocaine vaccine, a pool of mice immunized cocaine-CTB conjugate PS-5.53 were tested in a 10 competition ELISA. Different drugs were tested at varying concentrations for their ability to inhibit the binding of antibodies to cocaine-HEL. The panel of drugs tested included cocaine, benzoylecgonine (the major metabolite of cocaine); dopamine, serotonin, and norepinephrine (neurotransmitters); 15 methylphenidate and amphetamine (CNS stimulators); procainamide HCl (a cardiac depressant); atropine (a compound that has a tropane ring in its structure); and lidocaine (a general anesthetic). The pool of anti-cocaine antisera was specific for cocaine in that cocaine competed with the cocaine-HEL conjugate 20 for binding to the antibodies (Figure 23). Additionally, at high concentrations, benzoylecgonine, a cocaine metabolite, also bound to the antibodies. None of the other compounds were able to inhibit antibody binding to the conjugate.

25

EXAMPLE 22

Quantification of Cocaine-Specific Antibody

Without intending to limit the invention, one method of 30 directly quantifying the antigen binding capacity and affinity of the antigen-specific antibodies obtained using the cocaine conjugate vaccine is disclosed. The classical immunochemical technique of equilibrium dialysis is used. Immune sera elicited by immunization with cocaine-BSA PS-5.6 and control antisera 35 were placed inside dialysis bags (cellulose ester, 25,000 MWCO, Spectrum, Los Angeles, CA) and dialyzed to equilibrium against a

large volume containing various concentrations of 3 H-cocaine in PBS. This allowed measurement of the amount of cocaine bound to the antibody and the amount that was unbound. Data were analyzed both by plotting the amount of bound cocaine as a function of amount of total cocaine and by Scatchard plot (bound versus bound/free antigen). As expected, the antisera contained a heterogeneous mixture of antibodies with affinities ranging from 1×10^{-7} to -1×10^{-10} M. Measured cocaine binding capacity was up to about 10 μ M, indicating a concentration of antigen-specific antibody of about 0.7 mg/ml. Therefore, immunization with the cocaine conjugate vaccine can produce antibodies with a range of useful affinities and with high cocaine binding capacities, such that a substantial proportion of the total antibody in the circulation can react with and neutralize cocaine.

EXAMPLE 23

Efficacy of Cocaine-Specific Antibody in Inhibiting Cocaine Distribution in vivo

20

A. Inhibition of Cocaine Distribution to the Brain

To assess changes in cocaine tissue distribution caused by cocaine-specific antibody, 3 H-cocaine distribution was followed in PS-5.7 cocaine-BSA-immunized mice compared to BSA-immune control mice. Immune and control immunized mice were injected with 0.5 mg/kg 3 H-cocaine i.v. and then decapitated at 0.5 minutes after injection. Brains, hearts and blood (plasma) were removed for subsequent analysis of tissue and plasma cocaine concentration. Blood was collected into tubes containing sodium fluoride solution to inhibit esterases and containing EDTA to prevent clotting. Brains, hearts and plasma samples were placed into scintillation vials containing tissue solubilizer; digestion of samples occurred over 3 days at room temperature. The samples were decolorized and scintillation cocktail was added to each sample. Glacial acetic acid was added to clarify

the samples. After the samples were counted in a scintillation counter, data were converted to ng/g or ng/ml of tissue. Cocaine concentration in the brain tissue was significantly lower (n = 10, p < .05) at 0.5 minutes after injection (636.1 +/- 57.5 ng/g (mean +/- SEM) for cocaine-BSA-immunized vs. 1052.2 +/- 93.85 ng/g for BSA-immunized mice).

Cocaine concentration was also measured in the plasma. Cocaine-BSA-immunized mice had significantly higher (P<0.05) 10 levels of cocaine in the plasma (999.8 +/- 85.9 ng/ml) than did control BSA-immunized mice (266.5 +/- 51.0 ng/ml). The retention of cocaine in the plasma, due to antibody binding, could also be expressed as the apparent volume of cocaine in control (2.24 +/- 0.24 l/kg) and cocaine-BSA-immunized (0.53 +/- 0.04 l/kg) 15 mice. Measurement of the apparent volume of distribution provides a convenient way to determine whether the antibody levels are adequate to significantly affect the distribution of cocaine *in vivo*. Because it only requires measurement of plasma 20 levels of cocaine, it can also be used as a measure of antibody levels in humans following cocaine challenge.

Several groups of mice were injected two times i.v. with 0.5 mg/kg cocaine to determine the ability of cocaine-specific antibody to inhibit distribution of repeated doses of cocaine. 25 Only the second dose of cocaine, given 10 minutes after the initial dose, included the ³H-cocaine. The antibody inhibited the distribution of the cocaine redose to the brain tissue in cocaine-BSA-immunized mice (443.6 +/- 48.5 ng/g), compared to BSA-immunized mice (948.9 +/- 43.3 ng/g (n = 10, p < .001)). Thus, 30 the inhibition of cocaine distribution after the second dose of cocaine was similar to the inhibition of distribution after one dose.

35 B. Inhibition of Distribution to Cardiac Tissue

Immune and control immunized mice were anesthetized and intravenously injected with 0.015 mg/kg ³H-cocaine and were decapitated 0.5 minutes after injection. Brains, hearts and blood (plasma) samples were removed for subsequent analysis of cocaine concentration. The concentration of cocaine in heart tissue of cocaine-BSA immune mice at 5.7 +/- 0.78 ng/g was significantly lower than that of control BSA mice at 23.4 +/- 4.6 ng/g (n = 5, p < .001). The inhibition of cocaine distribution to heart tissue in cocaine-immunized mice was equal to or greater than the inhibition of cocaine distribution to brain tissue.

C. Inhibition of Cocaine Tissue Distribution After Intranasal
15 Administration

Effects of cocaine-specific antibody after intranasal cocaine administration were compared to effects after intravenous cocaine administration. In intranasal administration the 20 kinetics of distribution are different from intravenous administration. Immune or control mice were anesthetized and 1 mg/kg ³H-cocaine was intranasally administered in 50 μ l PBS. Cocaine levels did not peak until 2-5 minutes after intranasal 25 administration, as opposed to a 15 second peak after intravenous injection. Therefore, two minutes after cocaine injection mice were decapitated and brains and blood (plasma) samples were removed for subsequent analysis of cocaine concentration. In comparing intranasal cocaine administration to intravenous administration, total levels of cocaine in the brains of control 30 mice are fairly equal (1538 ng/g intranasally versus 2260 ng/g intravenously).

The distribution of cocaine to the brain after intranasal cocaine administration was inhibited by the presence of anti-cocaine antibody. Significant inhibition of brain distribution 35 of cocaine was measured after cocaine was intranasally

administered to cocaine-BSA-immune mice (708.3 +/- 82.8 ng/g), compared to control mice (1538.1 +/- 49.5 ng/g (n = 5, p < .0001)).

5

D. Antibody Titer

Mice with varying levels of cocaine-specific antibody were compared to determine how antibody titer may affect the level of inhibition of cocaine distribution. Groups of mice immunized in this study achieved titer levels ranging from 6,000 to 256,000. 0.015 mg/kg of ³H-cocaine was administered to mice having low titer (about 6,000 to 18,000) or high titer (about 54,000 to 256,000) anti-cocaine antibody. Thirty seconds after i.v. injection, mice were decapitated and brains and blood (plasma) samples were removed for analysis of cocaine distribution.

Mice with high antibody titers inhibited the distribution of cocaine to the brain highly significantly (control mice: 26.1+/- 2.0 ng/g, cocaine-immunized mice: 8.9 +/-1.2 ng/g; n= 10, p < .0001). In contrast, mice with low titers displayed a reduced ability to inhibit the distribution to the brain (control mice: 24.4+/-2.98 ng/g; cocaine immunized mice, 15.7+/-3.4 ng/g).

25 E. Cocaine Metabolism

To determine whether cocaine-specific antibody alters cocaine metabolism *in vivo*, cocaine metabolites were analyzed over time in cocaine-immune and control mice. Plasma samples tested were obtained as in animal experiments described and performed in Part A of this Example. The time point tested for metabolite composition was 30 minutes. The method for preparing the plasma for analysis is described above in Part A.

35 Plasma samples were aliquoted and non-radioactive cocaine, benzoyl ecgonine, and norcocaine were added in order to assist

in the UV visualization of the compounds. Samples were then applied to silica TLC plates which were developed in two solvent systems: methanol, chloroform, and triethylamine (3:1:0.1); and ethyl acetate, methanol, water, and concentrated ammonia (85:10:3:1). Metabolites were identified by reference to control compounds run on the same plates. The bands were scraped off the plates and ³H-containing compounds were detected through scintillation counting. From the counts obtained the amount of cocaine, benzoyl ecgonine, benzoic acid, and norcocaine as percent of total counts was determined. The total radioactivity in the plasma was determined by scintillation counting of whole plasma. Benzoic acid is detected as a metabolite when cocaine degrades into ecgonine methyl ester and benzoic acid, and so is equimolar to the ecgonine methyl ester metabolite.

The anti-cocaine antibodies appear to have minimal effects on cocaine metabolism *in vivo*. After 30 minutes the metabolites found are as follows, expressed as percent of total:

20

<u>Metabolite</u>	<u>Coc-BSA Immune</u>	<u>BSA Control</u>
Cocaine	19.66 +/- 7.5	17.31 +/- 3.7
Norcocaine	5.5 +/- 0.93	3.6 +/- 0.93
Benzoic Acid	47.51 +/- 8.5	50.28 +/- 4.4
Benzoyl	27.3 +/- 0.6	29 +/- 7.2
Ecgonine		

35

F. Disappearance of Cocaine From Plasma

In order to determine whether cocaine-specific antibody changes the rate of disappearance of cocaine from the plasma, 5 plasma samples collected at different times after cocaine injection in cocaine-BSA-immunized animals and in BSA-immunized animals were analyzed. Immune and control immunized mice were injected with 1 mg/kg ³H-cocaine i.v. and then decapitated at 0.5, 5 or 30 minutes after injection. Brains and blood (plasma) were 10 removed for subsequent analysis for brain and plasma cocaine concentration, percent of cocaine bound to antibody, and TLC for quantitation of cocaine and cocaine metabolites in plasma.

Plasma was analyzed as described above in Part E above for 15 percent of total radioactivity in the form of cocaine and any metabolites. Plasma samples were also analyzed for total radioactivity. The rate of disappearance of cocaine from the plasma of cocaine-BSA-immunized mice was compared to the rate of disappearance of cocaine from BSA-immunized mice. In this 20 analysis, the small fraction of norcocaine (less than 5%) was considered with the cocaine since norcocaine has CNS activity and binds to antibody. This does not alter the results described below.

25 Cocaine disappears from the plasma of both groups of animals at very similar rates. Between 30 seconds and 30 minutes, about 80% of the cocaine had disappeared from the plasma of both groups of animals. The disappearance of cocaine in plasma at these times after injection was due to both redistribution and metabolism. 30 Although cocaine disappears at a similar rate in the two groups of animals, there is more cocaine in the plasma of the cocaine-BSA-immunized mice than in plasma from the BSA-immunized mice at all times. The presence of cocaine-specific antibody did not detectably alter the elimination of cocaine.

G. Percent of Cocaine Bound to Antibody

The inhibition of distribution as shown above is possible if cocaine is bound to antibody in the animal. To determine the degree of binding of plasma cocaine to antibody, immune and control immunized mice were injected with 1 mg/kg ³H-cocaine i.v. and then decapitated at 0.5 minutes after injection. Blood (plasma) was removed and protein G beads were used to capture the antibody-cocaine complexes. Protein G beads were added to plasma from ³H-cocaine-injected animal (with NaF to inhibit cocaine degradation) and incubated. After rinsing, each of the rinse volumes and the beads were added to scintillation fluid. The ³H-cocaine was detected by scintillation counting. The same plasma was analyzed for degradation of cocaine as in the metabolism assay (Part E) above. Since the antibodies made after immunization with cocaine-BSA bind to cocaine and to norcocaine, but not to the other major metabolites, as demonstrated in the Examples, percent binding was calculated based on the amount of cocaine and norcocaine found in the plasma sample.

20

In the animals which were immunized with cocaine-BSA, an average of about 50% of the cocaine in the plasma sample was bound to antibody. This is compared to the BSA-immunized animals, in which 3% of the cocaine was bound to antibody. The 3% value represents the background in the assay.

H. Cocaine-CTB Hapten Carrier Elicits Effective Antibody

Cocaine-CTB PS-5.53 was injected into mice to determine whether it was able to elicit antibodies that would alter cocaine distribution. CTB itself was injected into groups of control mice. Mice were boosted with cocaine-CTB PS-5.53 and PS-5.70 as needed until the antibody titers were about 54,000 or greater. The methods used for immunization and assaying cocaine-specific antibody titers are described in Examples. Mice with cocaine-specific antibody titers and control mice were injected with 0.5

mg/kg 3 H-cocaine and were decapitated 30 seconds after injection. Brain tissue and plasma was isolated and analyzed for 3 H-cocaine content as described in part A of this Example.

The antibody produced after immunization with cocaine-CTB inhibited the distribution of cocaine to the brain significantly. For cocaine-CTB immunized compared to CTB-immunized mice there was significantly less cocaine in the brain tissue (678.8 ng/g compared to 885.4 ng/g, n=6, p=0.0004 by two-tailed t-test). Likewise, the cocaine was retained in the plasma of cocaine-CTB to a significantly greater extent than in the CTB-immunized animals. Therefore the cocaine-CTB is effective in generating antibody that will inhibit the distribution of cocaine to the brain.

EXAMPLE 24

15 Passive Transfer of Immune Immunoglobulin in Mice

Mice are immunized with PS-5-CTB using optimal immunization regimens as described in the Examples. At varying times, mice are bled and the titers of anti-cocaine antibody are assessed by 20 ELISA. Animals with antibody titers of about 54,000 or greater are sacrificed and bled by cardiac puncture. Control mice are immunized with the carrier protein alone. Sera from multiple mice (at least 20) are pooled and the IgG fraction is isolated by ammonium sulfate precipitation. Following dialysis to remove the 25 ammonium sulfate, the level of cocaine-specific antibody in the pooled immunoglobulin fraction is quantified by ELISA. Varying amounts of immunoglobulin are administered i.p. or i.v. to naive mice. After 24 hours, the recipient mice are bled and the serum assayed to determine the level of cocaine-specific antibody. 30 Using this method, the amount of antibody that must be transferred to achieve a given titer is determined. Groups of mice are given immune immunoglobulin and bled at varying periods of time to determine the clearance rate of the antigen-specific antibody. Other groups of mice are challenged with radiolabelled cocaine, as 35 described in the Examples, and cocaine distribution to the brain are measured. Control mice received IgG from carrier-immunized

mice. These experiments demonstrate the ability of passively transferred immune immunoglobulin to inhibit cocaine entry into the brain.

5 **EXAMPLE 25**

Passive Transfer of Immune Immunoglobulin in Humans

A pool of human donors is immunized with PS-5-CTB or other conjugates of the invention using optimal immunization regimens as 10 described in the Examples. At various times, donors are bled by venipuncture and the titers of anti-cocaine antibody are assayed by ELISA. Hyperimmune plasma from multiple donors is pooled and the IgG fraction is isolated by cold alcohol fractionation. The antibody preparation is buffered, stabilized, preserved and 15 standardized as needed for hyperimmune antibody preparations for human use. The level of anti-cocaine antibody is standardized by ELISA or other antibody-based assay.

An appropriate dose of purified antibody is administered to 20 patients intramuscularly or intravenously with or without the cocaine-CTB vaccine, but not in the same anatomical site as the vaccine. The appropriate dose is determined by assaying serum levels of recipients in a trial patient population by ELISA or other antibody-based assay at 24 hours or other appropriate time 25 point after injection of the hyperimmune antibody preparation and/or assaying the effectiveness of different doses in inhibiting cocaine effects.

The passively transferred immune globulin inhibits cocaine 30 effects in the patients. The use of human donors, polyclonal antibody, and the large number of donors in the donor pool limits the chance of immune response by the patients to the transferred antibody. This demonstrates that the cocaine-CTB elicits antibodies in a donor pool that can be used to passively immunize 35 patients against the effects of cocaine.

EXAMPLE 26

Preparation of Nicotine Conjugate

5

METHOD A

To a solution of nornicotine (50 mmol) in methylene chloride was added triethylamine (75 mmol), followed by succinic anhydride (100 mmol). The solution was heated at reflux for 18 hours. The 10 reaction mixture was washed sequentially with 10% aqueous hydrochloric acid, saturated sodium bicarbonate solution, brine and water. After drying ($MgSO_4$) and removal of the solvents under reduced pressure, the residue was purified using silica gel flash chromatography to furnish the desired product.

15

METHOD B

The succinylated nornicotine was then used to synthesize the nicotine conjugate PS-54 (Figure 18). To a solution of succinylated nornicotine (5 μ mol) in DMF (0.1 ml), 20 diisopropylethylamine (10 μ mol) was added followed by HATU (5.5 μ mol). After 10 minutes, the pale yellow solution was added dropwise to a solution of either HEL or BSA (500 μ g) in 0.1 M sodium borate buffer at pH 8.8 (0.9 ml) and the mixture stirred for 18 hours at ambient temperature. The pH of the conjugate 25 solution was adjusted to pH 7.0 by careful addition of 0.1 M aqueous hydrochloric acid, followed by purification by dialysis against PBS. The dialysate was filtered through a 0.2 μ m filter and the level of hapteneation measured by mass spectral analysis or UV absorbance.

30

Induction of Nicotine-Specific Antibody Responses

To induce an antibody response specific for a small molecule, or hapten, such as nicotine, it was necessary to link it to a T-cell epitope-containing carrier, e.g., a protein carrier. The 35 carrier is recognized by T-cells which are necessary for the

initiation and maintenance of antibody production by nicotine-specific B cells. In this example, the carrier used was BSA. A panel of structurally distinct nicotine-BSA conjugates was produced that were linked through different parts of the nicotine molecule with several different types of linkers (Fig. 17b). The set of different conjugates allowed the testing of different alterations and presentations of the nicotine molecule. Since any given nicotine conjugate may induce variable amounts of antibodies which recognize either the free hapten (nicotine), the carrier, or the conjugate only (and do not recognize nicotine itself), screening of the conjugates was performed as in the following example.

Mice were immunized intraperitoneally with 50 µg nicotine-BSA conjugate PS-55 in complete Freund's adjuvant. A second injection of PS-55 was given on day 21 and the mice were bled on day 35. Sera were tested in an ELISA for antibody binding to a conjugate of PS-55 and hen egg lysozyme protein (HEL) and are shown in Figure 24. These data demonstrate that this nicotine-BSA conjugate was able to induce strong antibody responses.

20

Recognition of Free Nicotine

To determine whether the induced antibodies are capable of recognizing the free nicotine molecule, a competition ELISA was performed. In this assay, free nicotine competes with PS-55 HEL coated to ELISA plates for the binding of antibodies in the sera. If the antibodies that have a high affinity for nicotine comprise most of the antibodies binding to the PS-55 HEL, then low concentrations of nicotine are capable of effectively inhibiting the antibody binding. For 3 out of 4 mice described above which were injected with PS-55 BSA, antibody binding to PS-55 HEL was inhibited by free nicotine (Figure 25). Note that the presence of antibody specific for the conjugate alone would not be expected to interfere with the action of the anti-nicotine antibody. This indicates that antibody is present in each of these sera that recognizes free nicotine. The major metabolite of nicotine, cotinine, was also tested in the competition ELISA and it cannot

compete with antibodies in any of the sera except at very high concentrations.

To verify that the induced antibodies were capable of recognizing the free nicotine molecule, an RIA was used to measure specific binding to [³H]-nicotine. Immune sera from the above experiment was incubated with [³H]-nicotine and protein-G-conjugated Sepharose beads (Gammabind-G Sepharose, Pharmacia), which bind IgG in the sera samples. The antibody-bound [³H]-nicotine was isolated by centrifugation of the beads and was detected by scintillation counting of the beads. Sera from 3 out of the 4 mice bound significantly to free [³H]-nicotine (Figure 25). Pre-incubation of these sera with 50-fold excess unlabeled nicotine completely inhibited the binding of the [³H]-nicotine to these antibodies. These data demonstrate that nicotine-carrier conjugates have been synthesized which induce nicotine-specific antibody responses that should be capable of preventing the distribution of nicotine to the brain *in vivo*.

Specificity of Nicotine-Specific Antibodies

To analyze the specificity of the anti-nicotine antibodies induced by the nicotine vaccine, sera from the mice immunized with nicotine-CTB conjugate are tested in a competition ELISA. A panel of metabolites of nicotine and related molecules are tested at varying concentrations. If the antibodies have high affinity for the metabolite, then low concentrations are capable of effectively competing this assay. The relative reactivity is expressed as the IC₅₀, the concentration of the inhibitor that decreases the ELISA signal by 50%. The following metabolites are tested for reactivity: nicotine glucuronide, cotinine, cotinine glucuronide, trans-3'-hydroxycotinine, trans-3'-hydroxycotinine glucuronide, nicotine 1'-N-oxide, cotinine N-oxide, and nornicotine.

Efficacy of Nicotine-Specific Antibody in Inhibiting Nicotine Distribution in vivo

Inhibition of Nicotine Distribution to the Brain

To assess changes in nicotine tissue distribution caused by nicotine-specific antibody, ³H-nicotine distribution is followed in nicotine-CTB-immunized mice compared to CTB-immune control mice. Immune and control immunized mice are injected with 0.02 mg/kg ³H-nicotine i.v. and then decapitated at 0.5 minutes after injection. Brains and blood (plasma) are removed for subsequent analysis of tissue and plasma nicotine concentration. Blood is collected into tubes containing EDTA to prevent clotting. Brains and plasma samples are placed into scintillation vials containing tissue stabilizer; digestion of samples occurs over 3 days at room temperature. The samples are decolorized and scintillation cocktail is added to each sample. Glacial acetic acid is added to clarify the samples. After the samples are counted in a scintillation counter, data are converted to ng/g or ng/ml of tissue. Nicotine concentration in the brain tissue of nicotine-³H-CTB-immunized mice is significantly lower after injection of ³H-nicotine than in brain tissue of CTB-immunized control mice.

Example 27

Method A *NOT GOOD*

25 ***N'*-Butyric acid adduct of (S)-Nicotine**

To a solution of (S)-nicotine (0.031 moles) in anhydrous methanol (50ml) at ice-water temperature under argon, ethyl-4-bromobutyrate (0.0341 moles) was added dropwise over 10 minutes. 30 The resulting orange colored solution was allowed to warm to ambient temperature and stirred for 18 hours. The solvents were removed under reduced pressure leaving a brown residue which was precipitated with hexane to give an analytically pure sample of the desired ester.

The ester (36mg) was dissolved in methanol (3ml) and 1M sodium hydroxide solution (5ml) and stirred for 18 hours at ambient temperature. The solvents were removed under reduced pressure and the residue dissolved in 10% hydrochloric acid and 5 extracted with ethyl acetate. Following drying ($MgSO_4$) the solvents were removed under reduced pressure to yield the desired compound.

10

Method B

N'-Valeric acid adduct of (S)-Nicotine

To a solution of (S)-nicotine (0.031 moles) in anhydrous 15 methanol (50ml) at ice-water temperature under argon, 1-bromovaleric acid (0.0341 moles) was added dropwise over 10 minutes. The resulting orange colored solution was allowed to warm to ambient temperature and stirred for 18 hours. The solvents were removed under reduced pressure leaving a brown 20 residue which was precipitated with hexane to give an analytically pure sample of the desired compound.

Method C

25

N'-Hexanoic acid adduct of (S)-Nicotine

To a solution of (S)-nicotine (0.031 moles) in anhydrous methanol (50ml) at ice-water temperature under argon, 1-bromohexanoic acid (0.0341 moles) was added dropwise over 10 30 minutes. The resulting orange colored solution was allowed to warm to ambient temperature and stirred for 18 hours. The solvents were removed under reduced pressure leaving a brown residue which was precipitated with hexane to give an analytically pure sample of the desired compound.

Method D*N'-Octanoic acid adduct of (S)-Nicotine*

5 To a solution of (S)-nicotine (0.031 moles) in anhydrous methanol (50ml) at ice-water temperature under argon, the appropriate 1-bromoocanoic acid (0.0341 moles) was added dropwise over 10 minutes. The resulting orange colored solution was allowed to warm to ambient temperature and stirred for 18 hours.

10 The solvents were removed under reduced pressure leaving a brown residue which was precipitated with hexane to give an analytically pure sample of the desired compound.

15

Example 28**Method A***General preparation of PS-55, PS-56, PS-57 and PS-58*

20

To a solution of the appropriate N'-alkanoic acid analog of nicotine (6.27×10^{-5} moles) (from Example 26) in DMF (1.6ml), DIEA (1.25×10^{-4} moles) and HATU (7.53×10^{-5} moles) were added. After 10 minutes at ambient temperature, the pale yellow solution was added

25 to either HEL or BSA (16.5mg) in 0.1M sodium bicarbonate, pH 8.3 (14.4ml) and stirred for 18 hours. The conjugate solution was purified by dialysis against PBS at 4°C overnight. The conjugates were analyzed using laser desorption mass spectral analysis to determine the number of haptens.

30

Method B*General preparation of PS-55, PS-56, PS-57 and
PS-58 with rCTB as carrier protein*

5 To a solution of the appropriate N'-alkanoic acid analog of
nicotine (6.27×10^{-5} moles) (from Example 26) in DMF (1.6ml), DIEA
(1.25×10^{-4} moles) and HATU (7.53×10^{-5} moles) are added. After 10
minutes at ambient temperature, the pale yellow solution is added
10 to rCTB (16.6mg) in 0.1M sodium bicarbonate, pH 8.3 (14.4ml) and
stirred for 18 hours. The conjugate solution is purified by
dialysis against PBS at 4°C overnight. The conjugates are
analyzed using laser desorption mass spectral analysis to
determine the number of haptens.

15

Example 29**Preparative-Scale Purification of rCTB**

20 rCTB from *V. cholerae* supplied from SBL Vaccin AB in 0.22 M
phosphate pH 7.3, 0.9% NaCl buffer was diafiltered into 20 mM
sodium phosphate, pH 6.5. A sample was then purified using cation
exchange chromatography on Pharmacia SP Sepharose Fast Flow resin
25 with Buffer A: 20 mM sodium phosphate pH 6.5 and Buffer B: 20 mM
sodium phosphate pH 6.5, 1.0 M NaCl as the elution buffers. The
purified fractions were analyzed by SDS-PAGE, staining with Daichi
Silver Stain. The purified sample was filtered through a 0.22
micron filter and stored sterile at 4°C.

30

Example 30
Method A (Analytical)

35 Samples for analytical reverse phase HPLC (RP HPLC) were
prepared by the following method: 100 μ l of conjugate CTB-5.200

was precipitated by adding 1.0 ml of absolute ethanol and freezing at -80°C overnight. The conjugate was spun at 14000 rpm for 20 minutes at 4°C and then the ethanol was decanted off and the pellet air dried. The pellet was resuspended in 25 µl of 20% acetonitrile with 0.1% trifluoroacetic acid (TFA) and protein concentration measured by the Pierce Micro BCA assay.

The conjugate was analyzed using a C18 reverse phase column (Vydac No. 218TP5215 narrow bore) 2.1 x 150mm; particle size: 5µ; flow rate: 200 µl/min.; Buffer A: 100% water 0.1% TFA; Buffer B: 80% acetonitrile, 0.08% TFA. The gradient started at 16B, increased to 56%B over a period of 50 minutes, increased to 80%B at 60 minutes, and was held for 10 minutes.

Method B (Semi-Preparative)

15 Samples for RP HPLC on the semi-preparative scale were prepared as follows: two vials of CTB-5.200 lyophile were resuspended in 20% acetonitrile 0.1% TFA, sterile filtered, and quantitated by the Pierce Micro BCA. Two injections of 1.24 mg 20 each were made on a semi-preparative RP HPLC system using a C18 column (Vydac No. 218TP1520) 10 x 50 mm, particle size: 5 µ; flow rate: 1.8 ml/min; Buffer A: 0.1% TFA in water; Buffer B: 0.08% TFA in 80% acetonitrile. A stepwise gradient was used as follows: 20%B for 10 minutes, 35%B for 40 minutes, 55%B for 5 25 minutes, finishing with a 5 minute wash out at 100%B. Peaks were collected and immediately lyophilized.

Example 31

Coorelation of GM1 Binding and Immunogenicity

30 An in vitro measurement of conjugate binding, the GM1 ELISA, was compared to *in vivo* immunogenicity for a panel of cocaine-CTB conjugates. The ganglioside GM1, the natural cellular ligand for cholera toxin binding, was coated onto ELISA plates. The conjugates were then incubated on the plates; only functional 35 multimers of the CTB are capable of binding to a GM1-coated plate. A cocaine-specific murine antibody pool was used to detect hapten

on the bound conjugates. The cocaine-CTB conjugates were then screened in an immunogenicity assay to determine if they induced anti-cocaine antibodies. In screening the cocaine-CTB conjugates there was a positive correlation between GM1 binding and immunogenicity (data not shown). Five out of eight cocaine-CTB conjugates that were positive in the GM1 ELISA (concentrations of less than 20 ng/ml are regarded as positive responses) also were positive in the immunogenicity assay (sera from 2 out of 3 mice tested at a 1/900 dilution generated an O.D. of 0.900 or higher).
10 Most of the conjugates that were not positive in the GM1 ELISA also were not positive in the immunogenicity assay.

Example 32

Level of Haptenation vs. Immunogenicity

15 The ratio of drug hapten to carrier protein in the conjugate may alter the ability of the conjugates to stimulate production of hapten-specific antibody. The conjugation reaction was altered to produce cocaine-CTB conjugates with several different levels of haptenation. Degree of haptenation was calculated by analysis of
20 mass spectrometry of the conjugates. These conjugates were screened for biological activity in immunogenicity experiments and by mass spectrometry analysis for haptenation levels. Conjugates were made by three different methods using different ratios of haptenation reagents compared to carrier protein (Example 6: Methods G & L, Methods H & M, and Method I). A comparison of
25 level of haptenation and immunogenicity was made. For these conjugates, higher levels of haptenation produce lower quantities of anti-cocaine antibodies. The conjugates that had a lower range of haptenation produced higher anti-cocaine antibody levels.
30 Therefore in order to produce high anti-cocaine antibodies a certain range of haptenation has been determined to be advantageous.

Example 33**Induction of Cocaine Specific Antibody Response
in a Second Animal Model**

Rats were injected with cocaine-CTB PS-5.189 conjugate to 5 determine whether the conjugate induces cocaine-specific antibodies in a second species. Wistar male rats were immunized with 10ug of cocaine-rCTB conjugate precipitated on alum intramuscularly and again bled 14 days after the second injection. Sera were tested in an ELISA using plates coated with PS-5.4 10 conjugated to HEL (hen egg lysozyme). The response of 5 individual rats per group are shown in direct ELISA (Figure 26). These data demonstrate that the cocaine-rCTB conjugates are able to induce antibody responses in rats.

To directly determine whether the antibodies generated in 15 rats are capable of recognizing the free cocaine molecule, a competition ELISA was established. Plates were coated with PS-5.4 HEL and incubated with the anti-sera at a dilution that represents the 50% point of the curve and varying concentrations of free cocaine as competitor. If antibody which has a high affinity for 20 free cocaine comprises much of the antibody that is capable of binding to cocaine-HEL, then small amounts of free cocaine will inhibit the binding of the antibody to cocaine-HEL. When cocaine-rCTB conjugate was used as an immunogen the binding of antibodies in the rat sera were inhibited by free cocaine (Figure 27). The 25 major metabolite of cocaine, benzoylecgonine, was also tested in the competition ELISA and none of the sera tested are able to be competed by benzoylecgonine. These data demonstrate that cocaine-carrier conjugates can be synthesized which induce cocaine-specific antibody responses in rats.

30

EQUIVALENTS

Those skilled in the art will recognize, or be able to 35 ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described

herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

5

10

15

20

25

30

35